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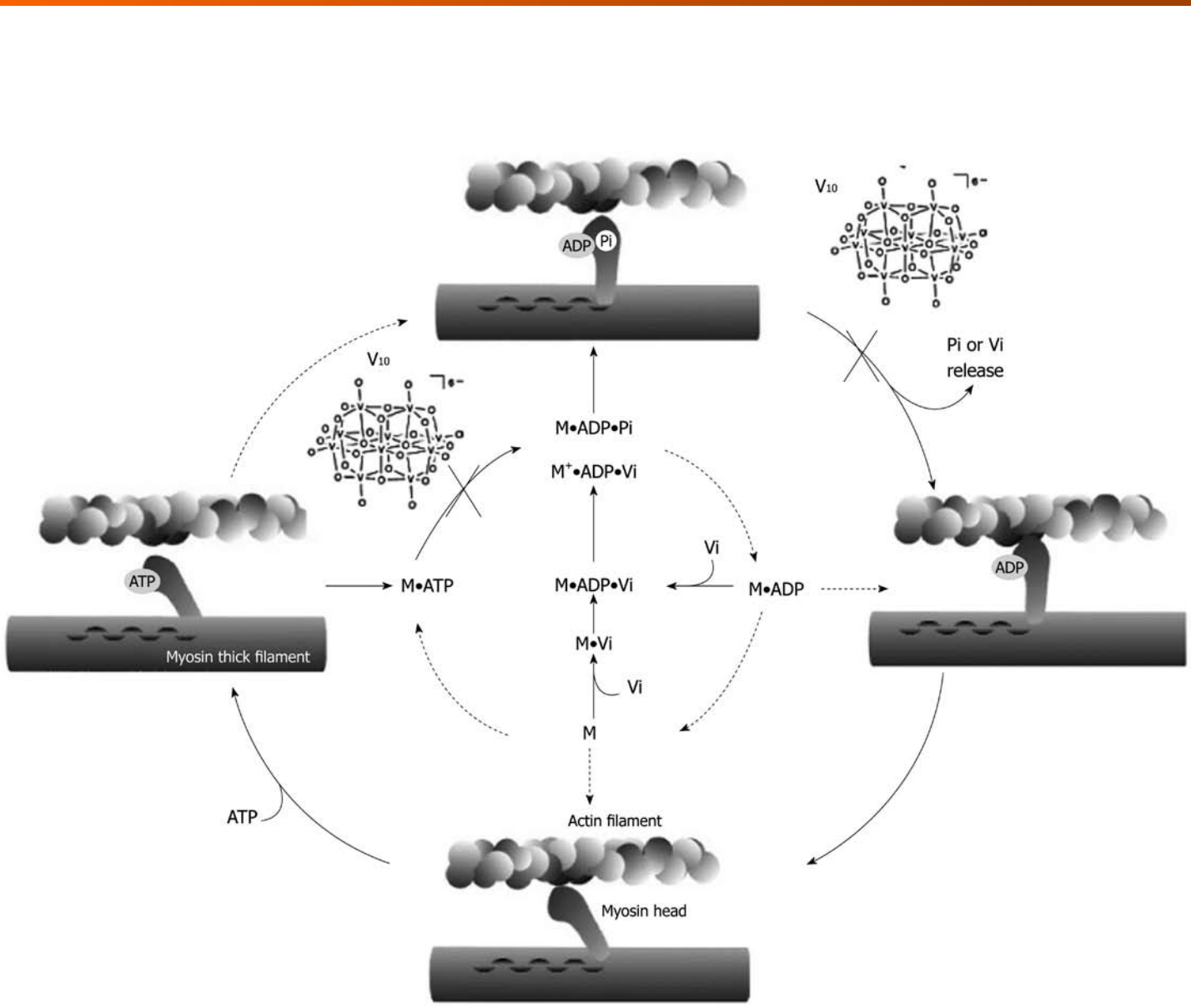

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EDITORIAL

Recent perspectives into biochemistry of decavanadate

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Abstract

The number of papers about decavanadate has doubled in the past decade. In the present review, new insights into decavanadate biochemistry, cell biology, and antidiabetic and antitumor activities are described. Decameric vanadate species (V_{10}) clearly differs from monomeric vanadate (V_1), and affects differently calcium pumps, and structure and function of myosin and actin. Only decavanadate inhibits calcium accumulation by calcium pump ATPase, and strongly inhibits actomyosin ATPase activity (IC_{50} = 1.4 μmol/L, V_{10}), whereas no such effects are detected with V_1 up to 150 μmol/L; prevents actin polymerization (IC_{50} of 68 μmol/L, whereas no effects detected with up to 2 mmol/L V_1); and interacts with actin in a way that induces cysteine oxidation and vanadate reduction to vanadyl. Moreover, *in vivo* decavanadate toxicity studies have revealed that acute exposure to polyoxovanadate induces different changes in antioxidant enzymes and oxidative stress parameters, in comparison with vanadate. *In vitro* studies have clearly demonstrated that mitochondrial oxygen consumption is strongly affected by decavanadate (IC_{50}, 0.1 μmol/L); perhaps the most relevant biological effect. Finally, decavanadate (100 μmol/L) increases rat adipocyte glucose accumulation more potently than several vanadium complexes. Preliminary studies suggest that decavanadate does not have similar effects in human adipocytes. Although decavanadate can be a useful biochemical tool, further studies must be carried out before it can be confirmed that decavanadate and its complexes can be used as anticancer or antidiabetic agents.

Key words: Decavanadate; Vanadate; Calcium pump; Myosin; Actin; Actin polymerization; Insulin mimetic; Antidiabetic agent; Antitumor agent

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INTRODUCTION

The number of articles about vanadium in the past decade (2001-2010) has doubled in comparison to the previous one (1991-2000), from 1149 to 2616, of which, 74 (48 in the previous decade) are about decavanadate. A relevant contribution towards our understanding of the effects of vanadium in the environment, biochemistry, biology and health was published in 1998[1]. In the past decade, at least five reviews on different aspects of vanadium have been published, covering chemistry, chemical engineering, biochemistry, biology, pharmacology and medicine[2-6], which is a testimony to the recent interest in this transitional metal in several scientific areas. However, there have been few studies about decavanadate and only seven *in vivo* studies have been published in the past decade[7-13]. These *in vivo* studies have demon-

strated that decavanadate in animals induces different changes in vanadium accumulation, lipid peroxidation and antioxidant enzyme activity than those observed for monomeric vanadate, and consequently it can also contribute to the effects described for vanadium. Therefore, the different changes in oxidative stress markers and lipid peroxidation, among others, can be attributed to decavanadate[7-13]. In several kinetic studies, following decavanadate administration, nuclear magnetic resonance (NMR) and UV/Vis spectroscopy have been used to correlate the vanadate species with the observed biological effects[7-13].

Decavanadate is well known to interact with several proteins and to have many biological activities, mainly *in vitro,* as recently reviewed[14,15]. The first enzyme reported to be inhibited by decavanadate was muscle adenylate kinase[16]. Other enzymes included hexokinase, phosphofructokinase and inositol phosphate metabolism enzymes[17,18]. In the past decade, it has been demonstrated in our laboratory that decavanadate interacts with calcium ATPase, myosin and actin, suggesting that it can affect several biological processes, such as muscle contraction and its regulation, actin polymerization, and calcium homeostasis[19-23]. We believe that in many studies using vanadate, decavanadate species will form, and therefore they will contribute eventually to the described biological effects[14,15]. Decavanadate can be more or less effective than the corresponding simple oxovanadates[14-18].

Since it was discovered that ATP from Sigma contained vanadium[24], vanadium has been used as a tool to understand several biochemical processes[14,15]. Moreover, vanadate, is actually accepted as a potent inhibitor of protein tyrosine phosphatase (PTP), a key enzyme in the insulin signaling pathway. PTP is described as one the main targets of vanadate as an insulin mimicking agent, promoting an increase in glucose uptake in several types of cells[25].

In the present review, we describe recent insights into the effects of decavanadate on muscle proteins, such as myosin, actin and calcium pump, as well as its toxicological effects *in vivo* and, more recently, its antidiabetic and anticancer effects. Some comparisons will be made with the vanadyl cation, the tetravalent form of vanadium, which, although in the majority intracellularly, is not the main focus of this review, and the reader is referred elsewhere[26,27]. Although we present new data about the interaction of vanadate, decavanadate and vanadyl with actin, the main purpose is to highlight recent insights into decavanadate biochemistry, which are not usually taken in account in biological studies of vanadium.

COMPLEX CHEMISTRY OF VANADIUM: CAN WE BE CERTAIN ABOUT WHICH VANADIUM SPECIES ARE INDUCING THE BIOLOGICAL EFFECTS?

Can the complex chemistry of vanadium explain the diversity of its biological effects? Even in 2011, many studies using vanadium clearly misunderstand its chemistry, which leads to wrong conclusions and research directions in an attempt to clarify the biological role of vanadium. The complexity of vanadium chemistry in aqueous solutions includes: (1) several oxidation states; (2) chemical similarity of vanadate and vanadium (V) with phosphate; (3) ability to form vanadate oligomers, such as decavanadate; (4) capacity to form complexes with many molecules of biological interest such as ATP, ribose, glutathione and amino acids, through different coordinating atoms such as oxygen, nitrogen and sulfur; (5) ability of having several geometric configurations, some mimicking enzyme substrate transition state analogs; (6) low solubility in aqueous solutions of some vanadium complexes of biological interest; and (7) low stability of many vanadium complexes used as insulin mimetics or anticancer agents under physiological conditions and at 37 ℃[3,5,6,14,15,18,28].

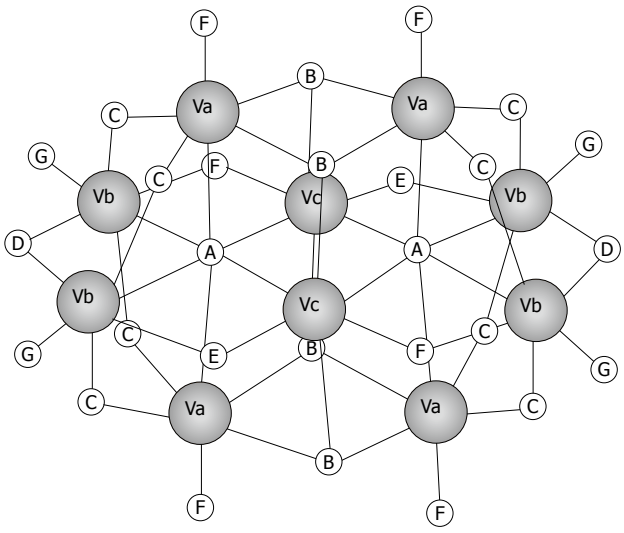

Figure 1 Schematic structure of V_{10} ($V_{10}O_{28}^{6-}$). Va, Vb and Vc represent the three different types of vanadium atoms described in the text.

Vanadate [vanadium (V)] generates a variety of different oxovanadates, depending on pH, concentration and specific conditions[26,29]. The simple vanadate colorless solution contains several metavanadate species (VO_3^-), depending on vanadium concentration, such as monomeric vanadate [V_1, orthovanadate species (VO_4^{3-})] dimeric vanadate (V_2), tetrameric vanadate (V_4) and pentameric vanadate (V_5)[15]. If, after preparation of a stock vanadate solution (for instance 10 mmol/L), acidification occurs, the instantaneous appearance of a yellow color indicates the formation of decavanadate, even if the overall pH value of the solution is not acidic, that is, does not change significantly[14,15]. Therefore, when using vanadate solutions in chemical, biochemical or biological studies, even at physiological pH values, it is critical to avoid acidification steps, unless decavanadate species are desirable[14,15]. Decavanadate, with a formula of $V_{10}O_{28}^{6-}$[15,30], has a unique structure, with dimensions of 8.3 Å, 7.7 Å and 5.4 Å (Figure 1). By ^{51}V NMR spectroscopy, three

different types of vanadium atoms can be distinguished (Va, Vb and Vc; Figure 1), whereas by UV/Vis spectroscopy, specific absorption at 360 and 400 nm, attributed to decavanadate species, can be detected, which is responsible for the typical yellow or bright orange of vanadium solutions[14,15,30]. In spite of this knowledge, many studies still misinterpret the chemistry of vanadium in solution particularly, and do not recognize that, if the vanadate solution turns yellow, this is due to the formation of decameric vanadate species[14,15]. Similarly, if the solution turns blue, this means that all the decameric vanadate has decomposed to the monomeric form of vanadate (colorless), followed by vanadium reduction to the vanadyl species that confers the blue color on the solution. Eventually, the observation of a green color during this process is due to the mixture of vanadyl (blue) and decavanadate (yellow) species.

Once formed even at neutral pH, decavanadate can be removed by two procedures; the most convenient method is to heat or boil the solution[31]. Alternatively, the solution can be aged. Depending on the pH, the decavanadate will ultimately convert to the colorless metavanadates or orthovanadates. As described above, it is important to recognize that generally a yellow color of these vanadate solutions reflects the fact that some decavanadate is present in the solution, and should be removed by heating if this species is not desired[31,32]. ^{51}V NMR spectroscopy can be used to monitor the speciation of oxovanadates in biological systems and experiments can be designed to evaluate specific interactions of the different vanadate oligomers with compounds in the biological system[33-35]. Decavanadate stability can be followed by UV/Vis spectroscopy, even for μmol/L concentrations, due to absorption in the ultraviolet region that confers the yellow color observed for decavanadate solutions[35,36].

Conversely, the lack of stability of some vanadium complexes used as antidiabetic or anticancer agents can contribute to misinterpretation about the role of vanadium in biology, namely its putative application as a therapeutic agent. In fact, in the majority of studies published describing the effects of vanadium complexes on biological systems, the authors have not taken into account the stability of vanadium complexes, which are often incubated with cells during long periods of time, and particularly at 37 ℃, which decreases vanadium complex stability. In fact, even vanadium complexes such as bis-maltolato-oxovanadium (IV) (BMOV), which is known for its insulin mimetic effects, decompose and are oxidized, even at 25 ℃[37]. By combining several spectroscopic techniques, it is possible to analyze the stability of the vanadium compounds and to confirm which species are truly present in the medium at the time they are promoting the observed effects, and even after inducing the effects. Therefore, without a clear demonstration that the vanadium species are present in the medium, and that the vanadium complexes or species have not decomposed, it can be only speculated that the observed effects are due to the vanadium compound that has been added to the medium.

DECAVANADATE INTERACTIONS WITH CALCIUM PUMP FROM SARCOPLASMIC RETICULUM

Sarco/endoplasmic reticulum calcium ATPase, a member of the E1E2 or P-type ATPase family, is present in two main conformations, E1 and E2, during the process of calcium translocation. It has been established that the E1 state is prevalent in the presence of $Ca^{2+}$and the E2 state in the absence of Ca^{2+}[38]. Moreover, E1 can be phosphorylated by ATP but not by inorganic phosphate, whereas E2 can be phosphorylated by inorganic phosphate but not by ATP. The catalytic site of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase contains an aspartyl residue that is phosphorylated by ATP during the catalytic cycle, forming an acyl phosphate anhydride[38]. In the E1 conformation, the protein captures Ca^{2+} from the cytoplasm and is phosphorylated by ATP to form E1-P(Ca), which then changes its conformation to E2-P with a concomitant loss of affinity for the Ca^{2+}, releasing it into the lumen. Subsequently, the enzyme phosphorylated in the conformation E2 suffers hydrolysis, then E2 turns into E1, and the cycle is again initiated[38,39].

Vanadate is well known as a specific inhibitor of the SR Ca^{2+}-ATPase[40-43]. Several kinetic studies have suggested that vanadate inhibits SR-ATPase by forming a transition state analog of the phosphorylated intermediate, blocking the E2 conformation of the protein[40]. Decavanadate also interacts with the SR calcium pump[41], at a distinct site from the phosphorylation site. Decavanadate can also interact with other protein conformations such as E1, E1-P and E2-P, contrary to monomeric vanadate, as described by ^{51}V-NMR spectroscopy[35,42]. Only decavanadate, and not vanadate, is able to inhibit calcium accumulation coupled with ATP hydrolysis in SR vesicles, as well as proton ejection by the (SR) Ca^{2+}-ATPase[35,42,43].

SR calcium pump has proven to be an excellent model to study toxicology effects of oxovanadates and vanadium complexes on E1E2-ATPases, such as the E1E2-Na^+, K^+-ATPase and Ca^{2+}-ATPase, once they are involved in essential ion homeostasis, such as Ca^{2+} homeostasis, therefore regulating several processes in muscle and non-muscle cells. The calcium pump from SR has previously been shown to address metals toxicity, once it was found to be inhibited by oxovanadates, such as decavanadate and tetrameric vanadate, vanadium citrate complexes and BMOV, among others[37,42-44]. Moreover, several conditions of calcium accumulation coupled or not coupled with ATP hydrolysis, can be addressed using vesicles from SR calcium ATPase and the effects of several oxovanadatates evaluated (Figure 2). The measurements of Ca^{2+} accumulation by the SR calcium pump can be performed when the calcium uptake is coupled with

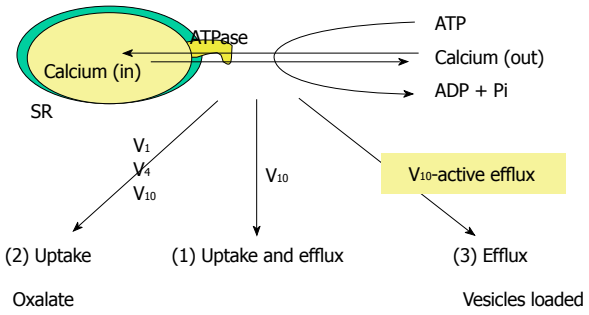

Figure 2 Modes of calcium translocation by SR calcium pump as affected by different vanadate oligomers. V_1: Monomeric vanadate; V_4: Tetrameric vanadate; V_{10}: Decameric vanadate. Only V_{10}, and not V_1, was shown to inhibit calcium uptake in conditions 1 and 3, that is, when ATPase activity is coupled to calcium transport. V_1 only inhibits the ATPase in condition 2, where the calcium gradient is destroyed by oxalate or phosphate.

ATP hydrolysis, therefore mimicking physiological conditions (Figure 2, condition 1, coupled uptake). At this condition, where a gradient of calcium modulated the calcium pump activity, only decameric vanadate (V_{10}) inhibits the calcium pump (Figure 2). In another condition, after filling the vesicles with calcium, when the efflux of calcium is coupled to ATP synthesis, it is observed that only decavanadate inhibits this process, whereas mmol/L concentrations of V_1 have no effect (Figure 2, condition 3, active efflux). In another different experimental condition, it is observed that, when the gradient of calcium is destroyed, meaning using phosphate or oxalate to reduce the calcium concentration inside the vesicles to almost zero, and the ATPase activity is at a maximum and we see mainly calcium uptake, calcium ATPase is inhibited by both V_{10} and V_1 solutions (Figure 2, condition 2, uncoupled calcium uptake)[19,32,35,43].

DECAVANADATE INTERACTIONS WITH SKELETAL MUSCLE MYOSIN

The mechanism of myosin ATPase inhibition by the monomeric vanadate species has been relatively well characterized; little has been reported about the inhibition of the process of muscle contraction by decavanadate. Whereas, monomeric vanadate (HVO_4^{2-}), mimics the transition state for γ-phosphate hydrolysis, at the active site[44], blocking myosin in a power-stroke state, by mimicking the ADP.Pi intermediate state, the decavanadate mode of action implies a binding site different from the ATP binding site. In fact, recent kinetic studies have shown that, unlike vanadate, decameric species were able to inhibit strongly the myosin or myosin subfragment-1 (S1) actin-stimulated ATPase activity with an IC_{50} of 6.11 ± 0.74 and 1.36 ± 0.14 μmol/L V_{10} for myosin and S1 (myosin subfragment S1, respectively, whereas no inhibitory effects were detected for vanadate up to 150 μmol/L[5,15,20]. A detailed kinetic analysis, revealed that decavanadate inhibition is non-competitive, yielding an inhibition constant Ki = 0.27 ± 0.05 mmol/L[20].

Another feature that distinguished the inhibition of the actomyosin complex by vanadate and decavanadate is the interaction of actin with myosin. Myosin-ADP-V_1 complex is destabilized by F-actin, inducing the release of the products, whereas myosin-MgATP-V_{10} is not. Therefore, only decavanadate prevents the release of the products during ATP hydrolysis by the actomyosin complex[20,21], inhibiting the stimulation of the myosin ATPase activity by actin. Apparently, decavanadate ($V_{10}O_{28}^{6-}$), induces the formation of the intermediate myosin-MgATP-V_{10} complex blocking the contractile cycle, most probably in the pre-hydrolysis state[20,21]. Although many aspects of the interaction of decavanadate with the process of ATP hydrolysis by the actomyosin complex is not completely understood, we infer that different oxovanadates are able to populate different conformational states of the myosin ATPase cycle depending on their oligomerization state. It is proposed that decavanadate inhibits myosin ATP hydrolysis, as well as F-actin stimulation of the release of the products, blocking ATP hydrolysis by the actomyosin complex, probably in the pre-hydrolysis state or before the interaction between actin and myosin, as shown schematically (Figure 3).

The walker A motif (corresponding to the P-loop in myosin) of ATP-binding cassette ATPases, is an anion-binding domain that can bind decavanadate with high affinity[45]. With myosin, decavanadate interacts with the phosphate-binding domains, in the vicinity of the nominated "back-door" binding site, interfering with movements associated with ATP hydrolysis by the actomyosin complex, therefore, by forming the intermediate myosin-MgATP-decavanadate complex[5,15,20,22]. The interaction of myosin with vanadate and decavanadate have mainly been described *in vitro* using skeletal muscle myosin (type Ⅱ myosin), whereas studies with non-muscle myosins, with cells or muscle fibers, using decavanadate have been scarce or non-existent. Some studies have reported the effects of vanadate in muscle fibers[46,47].

DECAVANADATE INTERACTIONS WITH SKELETAL MUSCLE ACTIN

To the best of our knowledge, before the past decade, only three studies were performed to investigate the interaction of vanadium with actin[48-50]. Vanadate has been shown to increase actin-actin interactions similarly to phosphate[48], and it also induces distinct effects on actin polymerization rather than phosphate[49]. Another study has analyzed vanadyl [vanadium (IV)] interaction with the monomeric actin, G-actin, revealing the presence of one strong vanadium binding site[50,51].

Contrary to myosin, not much information is available at the molecular level about decavanadate interaction with actin. The first study to describe the interaction between decavanadate and actin has suggested that actin, under certain experimental conditions, stabilizes the decomposition of decavanadate by increasing the half-life from 5 to 27 h, whereas no effects are detected upon myosin[36]. Moreover, it has been reported that decavana-

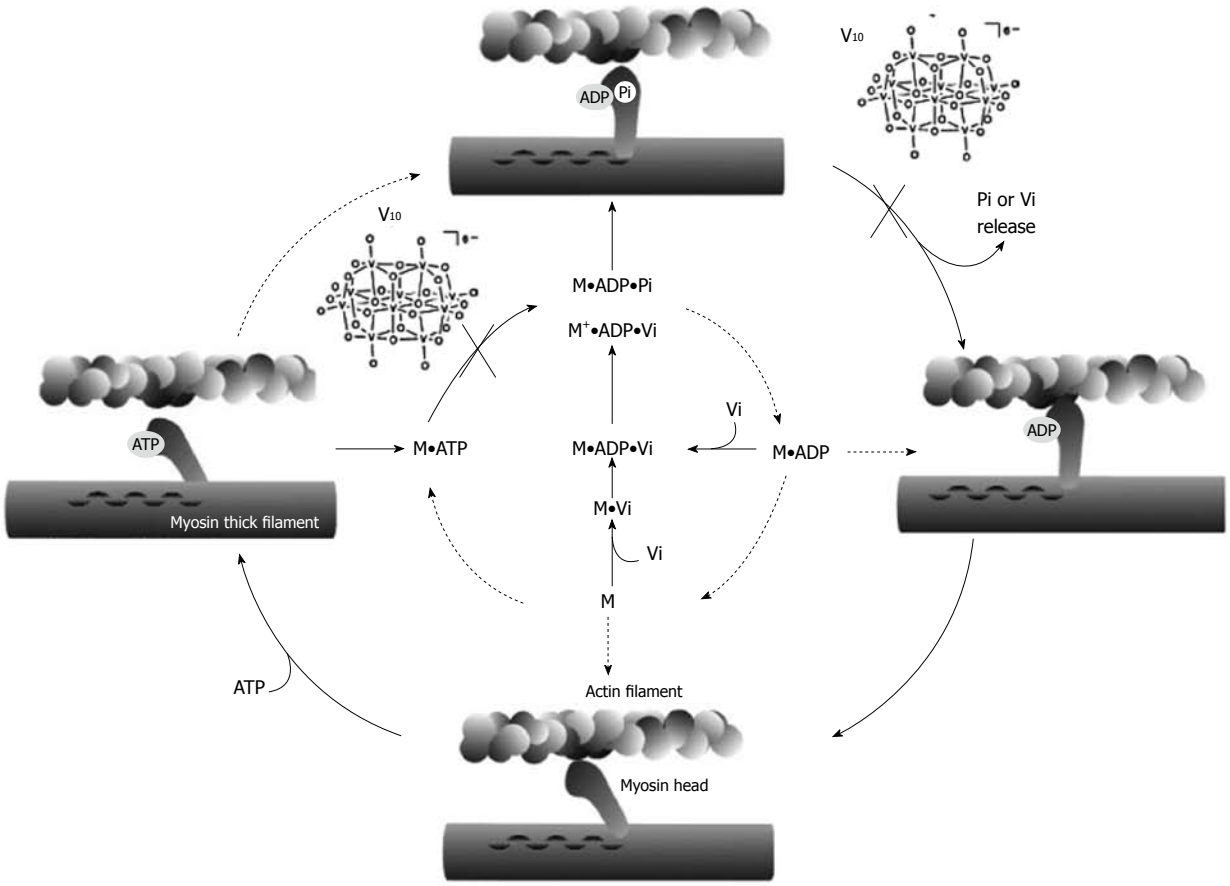

Figure 3 Relevant steps of ATP hydrolysis by actomyosin complex. The dominant process of ATP hydrolysis observed *in vitro* is indicated by the filled arrows. M: Myosin; Vi: Orthovanadate; V_{10}: Decavanadate. V_{10}, can blocked the process at two steps, without or with F-actin: before ATP hydrolysis and before product release, just before power stroke.

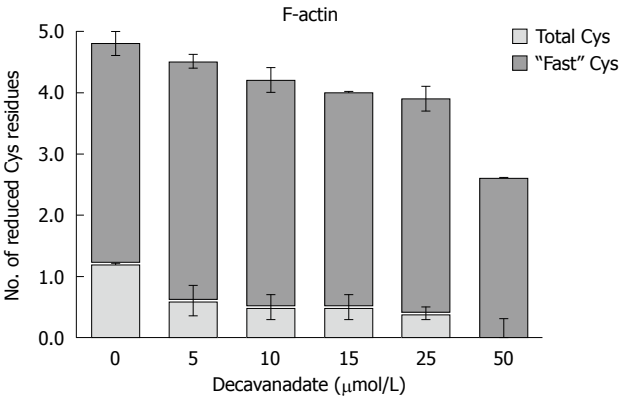

Figure 4 F-actin cysteine redox state, after 20 min exposure to decavanadate. Titration of cysteine was performed with 0.1 mmol/L 5,5'-ditiobis-(2-nitrobenzoic acid) and 2 μmol/L actin in 2 mmol/L Tris (pH 7.5), and 0.2 mmol/L $CaCl_2$. The increase in absorbance at 412 nm was continuously recorded over 10 min; To measure total cysteines the samples were treated afterwards with 1% SDS, and the absorbance was measured, over 15-30 min, until a steady value was reached. Titration with decavanadate produced a dose-dependent decrease in F-actin total cysteines, while Cys-374 (also named "fast cysteine") is reduced. The results shown are the average of triplicate experiments.

date inhibits the rate of G-actin (monomeric form of actin) polymerization into F-actin (polymerized form of actin), with an IC_{50} of 17 μmol/L[36], suggesting that it affects cytoskeleton structures responsible for many biologically significant processes. It has recently been reported that the interactions of decavanadate with actin induce protein cysteine oxidation and vanadate reduction[23,52].

It has been observed that only V_{10} solution, but not vanadate, is able to oxidize F-actin Cys-374 (also named "fast Cys") and one of the protein core cysteine residues (Figure 4), whereas for G-actin, only the latter effect is observed[52]. As described using NMR spectroscopy, ATP protects the actin from interaction with decavanadate, and prevents cysteine oxidation[23,52]. It has been demonstrated that decavanadate interactions with actin are of particular interest once it was observed that only V_{10} species are able to promote protein cysteine oxidation. However, does actin cysteine oxidation imply decavanadate reduction to vanadyl? In fact, decavanadate interaction with both G- and F-actin results in concomitant reduction of vanadate to vanadyl [vanadium at oxidation state (IV)][23,52]. Typical EPR vanadium (IV) signals can be detected upon decavanadate incubation with actin, whereas the presence of ATP in the medium once again prevents decavanadate reduction to vanadyl, as recently described[23,52]. EPR titration of vanadyl with G-actin shows that vanadyl binds to actin with a K_d of

Table 1 Decavanadate *in vivo* studies in 2001-2010

Tissue	Effects	Administration mode	Exposition time	Ref.
H	Antioxidant enzymes	ip	1, 7 d	[7]
H/K/L	Histological effects	ip	1, 7 d	[8]
L	Vanadium accumulation Antioxidant enzymes	iv	12, 24 h, 7 d	[9]
H/B	Vanadium accumulation	iv	1, 6, 12 h	[10]
H	Lipid peroxidation Antioxidant enzymes	iv	1, 6, 12 h	[11]
H	Vanadium accumulation Antioxidant enzymes	iv	1, 7 d	[12][1]

[1]Comparison between vanadium (decavanadate, vanadate) and cadmium (5 mmol/L) administration. H: Heart; K: Kidney; L: Liver; B: Blood.

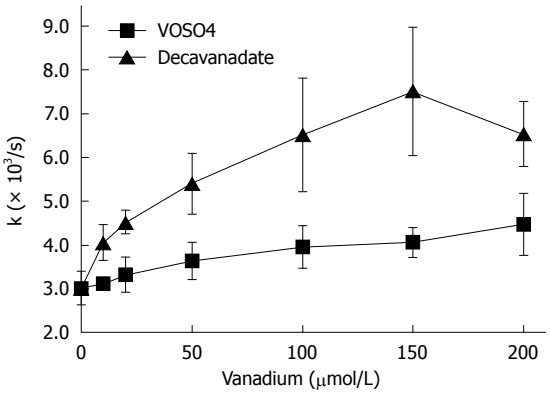

Figure 5 Exchange of bound ε-ATP of G-actin with ATP. Actin monomers (5 μmol/L) were incubated for 20 min with 0-200 μmol/L decavanadate, in 2 mmol/L Tris-HCl (pH 7.5), 0.2 mmol/L $CaCl_2$. The nucleotide exchange was monitored by the fluorescence decrease (λex = 350 nm; λem = 410 nm), as ε-ATP was replaced by ATP. Data are plotted as mean ± SD. The results shown are the average of triplicate experiments.

7.48 ± 1.11 μmol/L for G-actin and 43.05 ± 5.34 μmol/L for F-actin, with stoichiometry of approximately one and four vanadyl (VO^{2+}) cations bound per G- or F-actin molecule, respectively[52]. As described above for decavanadate, ATP prevents the interaction between vanadyl and actin, and therefore the observation of vanadyl EPR signals[52]. Both vanadyl and decavanadate interact with actin, but it has been shown that they induce different effects on protein structure and function, such as on myosin ATPase activity stimulated by F-actin, and actin polymerization, whereas decavanadate induces more potent effects on these two processes[23,52,53]. The effects of both vanadium species on actin structure have been compared, such as in protein intrinsic fluorescence, ATP exchange rate, and protein hydrophobicity[23,52,53]. It has been observed that decavanadate induces a more pronounced effect on the rate of ATP exchange rate, denoting a more open active site binding cleft (Figure 5). Decavanadate and vanadyl (up to 200 μmol/L total vanadium) both increased ε-ATP exchange rate ($k = 6.5 \times 10^{-3}$/s and 4.47×10^{-3}/s, respectively, in comparison with the controls: $k = 3.0 \times 10^{-3}$/s)[23,50,51], which clearly supports structural alterations to the actin ATP binding site.

TOXICOLOGY OF DECAVANADATE: *IN VIVO* AND *IN VITRO* STUDIES

In vivo studies of decavanadate administration have been performed since 1999, to understand the contribution of decameric vanadate species to vanadate toxicological effects[7-13]. Several experimental conditions have been used: (1) mode of decavanadate administration (intraperitoneal and intravenous); (2) fish species [*Halobatrachus didactylus* (*H. didactylus*)- Lusitanian toadfish - and *Sparus aurata* - gilthead seabream]; (3) vanadate concentration (1 and 5 mmol/L); (4) tissues (cardiac, hepatic, renal and blood); (5) subcellular fractions (cytosol, mitochondria, red blood cells and blood plasma); and (6) exposure time (1, 6, 12 and 24 h, and 2 and 7 d) (Table 1). A vanadate solution, not containing decameric vanadate species, was always administered for comparison. Following *in vivo* administration of decavanadate and vanadate solutions, several parameters were analyzed, such us: (1) vanadium subcellular distribution[7,9-11]; (2) histological changes in cardiac, hepatic and renal tissues[8]; (3) effects on SR Ca^{2+}-pump[13]; (4) lipid peroxidation; and (5) antioxidants enzyme activity and several oxidative stress markers in the heart[7,9,11] and liver[9]. It has been demonstrated that antioxidant stress markers, lipid peroxidation and vanadium subcellular distribution are dependent on the nature of the oxovanadates present in the administration fluid[7-13]. These studies have shown that, upon decavanadate administration, many effects are found that are not observed with vanadate, and conversely, many effects of vanadate are not observed with decavanadate.

Among several differences described, superoxide anion radical ($O_2^{\cdot-}$) production in mitochondria shows a dramatic difference upon decavanadate administration in comparison to vanadate[12]. $O_2^{\cdot-}$ production decreased by 35% in decavanadate-treated fish, whereas vanadate administration increased the $O_2^{\cdot-}$ production by 45%[12]. It should be noted that fish are very good models and adequate for these studies, because the physiological animal temperature (20-22 ℃) prevents decavanadate decomposition, and therefore, the effects can be seen. Therefore, pronounced increase of reactive oxygen species (ROS)

occurs in cardiac mitochondria following intravenous vanadate exposure, whereas decavanadate administration seems to prevent this effect. In *H. didactylus*, decavanadate (5 mmol/L, intraperitoneal) also induces a decrease in cardiac mitochondrial catalase activity (-60%) after 7 d. Taken together, these studies demonstrate that decavanadate exerts marked *in vivo* effects, with reactivity different from that obtained with simple vanadate.

Some of the above *in vivo* studies have demonstrated that following decavanadate administration, the mitochondrial fraction tends to accumulate more vanadium[7,11]. Moreover, decavanadate has specific effects on mitochondrial antioxidant enzyme activities[10,11]. Once again, these results confirmed that decavanadate behaves differently from vanadate. However, further studies will be required to clarify the importance of decavanadate for the biological effects of vanadium. We hope that others research groups will follow this direction.

During the studies described above, it has been observed that mitochondria accumulate vanadium, particularly when decavanadate is administrated. To explore further this association between mitochondria and decavanadate, studies were performed *in vitro*. In both hepatic and cardiac mitochondria, decavanadate inhibits mitochondrial respiration and induces mitochondrial membrane depolarization to a larger extent than monomeric vanadate[54]. For instance, decavanadate concentration as low as 100 nmol/L, inhibits 50% of oxygen consumption in mitochondria, while a 100-fold higher concentration of V_1 (10 μmol/L) is needed to induce the same effect. Moreover, decavanadate also induces mitochondrial depolarization (IC_{50} = 0.5 μmol/L) much more strongly than vanadate (IC_{50} = 50 μmol/L). These studies support the possibility that mitochondria are a potential cellular target for decavanadate[11,54,55]. Besides these mitochondrial effects of decavanadate (Table 2), in the past decade, several studies have shown that decavanadate has specific targets and many biological activities[5,14,15].

It was previously suggested that decameric vanadate species may eventually occur intracellularly in the cytosol, which is not acidic, upon acidification promoted by a chemical reaction or by an ionic pump[56]. However, based on vanadate chemistry, interconversion can occur in acidic compartments such as endosomes and lysosomes[14]. Therefore, the compartmentalization of different pH-containing domains in the cell favors the formation of decameric vanadate species[14]. After formation, decavanadate binds to specific protein binding sites, thus inducing different cellular responses from those of the other vanadate species (Figure 6). Therefore, a role of decameric vanadate species in biological chemistry is suggested[5,14,15].

Another potentially interesting feature of the effects of vanadate and decavanadate within cells is while the cytosol is at neutral pH, the membrane-bound intracellular compartments of the endocytic and secretory pathways are acidic. Therefore, the mode of entry into the cell plays a role in whether decavanadate is formed from monomeric vanadate (V_1). This further suggests that different cellular compartments might be differentially exposed to decavanadate. Although, the compartmentalization of vanadate species in cells is a subject still to be clarified, it has been proposed that V_{10} can be formed in acidic compartments in cells treated with vanadate, and ultimately is extruded into the medium. This confirms the possibility that V_{10} forms intracellularly[14]. Once outside the cells, decavanadate can cross membranes through specific anionic channels (Figure 6).

RECENT INSIGHTS INTO DECAVANADATE BIOLOGICAL AND BIOMEDICAL APPLICATIONS: INSULIN MIMETIC AND ANTITUMOR AGENT

Although many researchers remain skeptical whether decavanadate has a physiological role, in the past decade, several contributions have demonstrated that decavanadate induces relevant biological activities, which may eventually have a relevant impact in medicine (Table 2). By 2011, several studies about new decavanadate complexes, as well other polyoxometalates, have been published, and the potential medical applications are increasing, namely as insulin mimetic agents, inhibitors of aggregation of amyloid β-peptides associated with Alzheimer's disease, and as antitumor agents[5,14,15,57-61].

It is estimated that, by 2025, about 300 million people will have diabetes mellitus. Diabetic patients are also subject to other pathologies such as nephropathy, and arterial and neurodegenerative diseases. Vanadium, is well known to have insulin like or insulin-enhancing effects in several animal model systems[29,62-64]. These effects are probably induced through the inhibition of PTPs, as described above. However, vanadium may also, eventually, take action through ROS generation, and it is well known that transitional elements, such as vanadium, promote Fenton-like reactions. These actions could explain, at least in part, the antitumor effects of vanadium[65]. As an antidiabetic agent, vanadium has been described to act through an insulin-dependent or -independent pathway[29,62-66], although the mechanisms of action are still to be clarified.

Select polyoxometalates have been found to have insulin-enhancing properties[67], and recently, we have reported that the effect of decavanadate on glucose uptake in rat adipocytes was sixfold greater than the control level, and was more effective than BMOV and other vanadium complexes[68]. However, preliminary studies in human adipocytes (unpublished data) have shown that the effects described in rat adipocytes cannot be extrapolated to humans, after no similar effects were detected on glucose accumulation[69]. Several studies using decavanadate complexes have promoted the use of polyoxometalates as a tool for the understanding of many biological processes, including as antidiabetic and antitumor agents[5,14,15,57-60]. Medical applications of vanadium have been promoted in studies focusing on the structure-activity relationship

Table 2 Decavanadate *in vitro* studies in 2001-2010

Protein/effect	Vanadate species	Yr	Ref.
DNA-binding protein	V_{10}	2002	[70]
Methemoglobin reductase inhibition	V_{10}	2003	[73]
Actomyosin ATPase inhibition	V_{10}	2004	[20]
Muscle contraction regulation	V_{10}	2004	[21]
ATP sensitive cation -channel	V_{10}	2004	[76]
TRPM4 cation channels	V_{10}	2004	[71]
G-Actin polymerization inhibitor	V_{10}, V_4	2006	[36]
RNA triphosphatase	V_{10}	2006	[72]
P2X receptor antagonist	V_{10}	2006	[74]
Insulin mimetics	V_{10} compounds	2007	[57]
Back-door binding to myosin	V_{10}	2007	[22]
Porin (VDC) modulator	V_{10}	2007	[75]
Mitochondrial membrane depolarization	V_{10}, V_1	2007	[54]
Mitochondrial oxygen consumption	V_{10}, V_1	2007	[55]
Extracellular matrix mineralization	V_{10}, V_1	2008	[77]
Cardiomyocytes necrotic cell death	V_{10}, V_1	2008	[78]
Gelatine-mixtures	V_{10}	2008	[79]
Adipocytes glucose accumulation	V_{10}	2009	[68]
Actin oxidation and vanadyl formation	V_{10}	2009	[52]
Anticancer activity	V_{10} compounds	2009	[58]
ATPase activity in synaptic membranes	V_{10}	2009	[80]
Membrane models interaction	V_{10}	2009	[81]
DNA cleavage	V_{10}	2010	[82]
Actin structure and function	V_{10}	2010	[2]
Anticancer activity	V_{10} compounds	2010	[59]

TRPM4: Transient receptor potential cation channel subfamily M member 4.

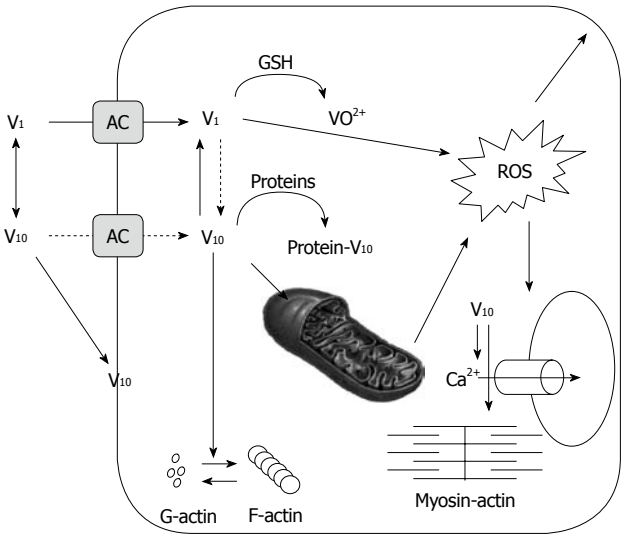

Figure 6 Scheme of proposed decavanadate (V_{10}) cellular targets. V_{10} uptake through anionic channels (AC). Decavanadate might interact with membrane proteins. V_{10} formation upon intracellular vanadium acidification in cytosol, but most probably in acidic organelles. Reduction of monomeric vanadate (V_1) by antioxidant agents. Binding of V_{10} to target proteins; it is proposed that V_{10} accumulates in subcellular organelles, such as mitochondria, affecting its function. Decavanadate also targets the contractile system, and its regulation, as well as calcium homeostasis (adapted from[14]).

of antidiabetic vanadium complexes, and vanadium compounds as antitumor drugs, to make vanadium available and safe for clinical use.

As described above, the *in vivo* studies of decavanadate administration in fish models took into account that decavanadate is sufficiently stable, and therefore the biological effects can be revealed. In studies with other animal models, and at different physiological temperatures, decavanadate stability will be different. In fact, it has been verified *in vivo* that decavanadate, at room temperature, has a half-life in serum of 15 h[7]. Moreover, in kinetic *in vitro* studies, performed at 25 ℃ or 37 ℃ (mitochondria studies) the half-life was 12 and 3 h, respectively[7-9,78]. The kinetic studies were always performed using a reaction time much less than the stability of decavanadate, between 10 to 30 min, to ensure that the biological effects were mainly due to decavanadate[7-13,52]. Therefore, the stability of the vanadate species is a very important factor in the biological effects of decavanadate. However, as described above, in the majority of the vanadium studies, the stability of the vanadate species or the vanadium complexes was not taken in account. In these studies, we can only speculate that the observed effects might have been due to the vanadium compound. Even for vanadium complexes, which are known to induce several insulin mimetic effects, it was verified that other species can be formed, even with other vanadium oxidation states than the original one[37]. Therefore, besides the factors described for vanadium complex chemistry, we may add the importance of certifying the stability of the vanadium complexes or species before attempting to attribute to them a certain biological activity or effect.

CONCLUSION

These studies have revealed the biological chemistry of decavanadate; a vanadate oligomer that eventually occurs in the cytoplasm more often than expected. Specific decavanadate interactions have been clearly demonstrated for myosin, calcium pump and actin, which are major proteins in muscle contraction and its regulation. Of particular interest are the V_{10} myosin back-door inhibition and the reduction of decavanadate by actin, although both processes still require to be clarified completely. Moreover, decavanadate inhibits strongly mitochondria, and therefore, cellular bioenergetics. In rat adipocytes, decavanadate can be a more potent insulin mimetic agent than BMOV, but preliminary results have shown a lack of effect in human adipocytes. It is proposed that the biological effects of vanadium may be explained, at least in part, by the capacity of decavanadate to induce many biological effects, some with medical applications.

In the present decade, we expect that important questions will be answered. (1) Will we be able to characterize the first X-ray structures of decavanadate-actin and decavanadate-myosin complexes? (2) Will we understand the role of decavanadate in the several steps of the process of actin polymerization/depolymerization? (3) Will we be able to understand the contribution of decavanadate as an insulin mimetic and anticancer agent? and (4) Will we be able to observe decavanadate formation in differ-

ent subcellular domains? These and others questions will require continuous development of new techniques and approaches to explore the vanadium effects in biology and their medical applications.

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TOPIC HIGHLIGHT

Hui-Ling Chiang, PhD, *Series Editor*

Survival and death of endoplasmic-reticulum-stressed cells: Role of autophagy

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Abstract

Accumulation of unfolded proteins in the endoplasmic reticulum (ER) results in ER stress, which subsequently activates the unfolded protein response that induces a transcriptional program to alleviate the stress. Another cellular process that is activated during ER stress is autophagy, a mechanism of enclosing intracellular components in a double-membrane autophagosome, and then delivering it to the lysosome for degradation. Here, we discuss the role of autophagy in cellular response to ER stress, the signaling pathways linking ER stress to autophagy, and the possible implication of modulating autophagy in treatment of diseases such as cancer.

Key words: Endoplasmic reticulum stress; Autophagy; Apoptosis; Cell survival; Cell death

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INTRODUCTION

The endoplasmic reticulum (ER) is an organelle that has essential roles in multiple cellular processes, including intracellular calcium homeostasis, protein secretion and lipid biosynthesis; all of which are required for cell survival and normal cellular functions. Normal ER functions are required for correct folding of newly synthesized proteins and their post-translational modifications, such as glycosylation and disulfide bond formation[1]. ER stress occurs in response to a variety of stimuli, various physiological and pathological conditions that can cause the accumulation of unfolded and misfolded proteins in the ER. Consequently, unfolded protein response (UPR) is triggered to resolve the ensuing stress by activating intracellular signal transduction pathways. In eukaryotic cells, UPR is mediated by three ER membrane-associated proteins, namely, inositol requiring enzyme (IRE)1α, PKR-like eukaryotic initiation factor (eIF)2α kinase (PERK), and activating transcription factor (ATF)6. These ER membrane-associated proteins are inhibited under basal conditions by their association with the chaperone protein Grp78/Bip, but are activated when released from Grp78 during ER stress[2]. These proteins induce signal-transduction events that can alleviate the accumulation of misfolded proteins in the ER by enhancing the protein folding capacity of the ER, by inhibiting new protein synthesis, or by accelerating the degradation of proteins. However, if the function of ER cannot be re-established,

extensive or sustained ER stress will eventually induce cell death through activating apoptosis. The phosphorylation of eIF2α at Ser51 by PERK during ER stress down-regulates efficient translation of most mRNAs, thereby inhibiting protein synthesis. Under these stressful conditions, only selected mRNAs such as ATF4, are translated. ATF4 induces expression of genes involved in restoring ER homeostasis[1,3]. ATF6 is transported to the Golgi in response to ER stress, where it is cleaved by Golgi-resident proteases S1P (site 1 protease) and S2P (site 2 protease). The cleaved ATF6 N-terminal fragment migrates to the nucleus to activate the transcription of UPR target genes[4]. Under conditions of ER stress, IRE1 processes X-Box binding protein (XBP)1 mRNA to generate mature XBP1 mRNA. Spliced XBP1 mRNA encodes a transcription activator that drives transcription of genes such as ER chaperones, whose products directly participate in ER protein folding. XBP1 also regulates a subset of UPR genes that promotes ER-associated degradation of misfolded proteins and ER biogenesis[5]. In addition to activation of the UPR by the pathways mentioned above, ER-stress leads to a release of Ca^{2+} from the ER into the cytosol, which, in turn, can activate signaling pathways involved in apoptosis and autophagy.

Autophagy is a lysosomal pathway responsible for the degradation of long-lived proteins, cellular macromolecules and subcellular organelles. Autophagy process involves the formation of double-membrane autophagic vacuoles, known as autophagosomes, which transport cytoplasmic cargo to the lysosome for degradation. Autophagy is induced during starvation in both yeast and higher eukaryotes as a way of breaking down macromolecules to recycle their components[6,7]. Autophagy is also involved in removing damaged or excess organelles. Autophagic activity is controlled by a set of evolutionarily conserved autophagy-related proteins (Atg proteins). The initial nucleation and assembly of the primary autophagosomal membrane requires a kinase complex that consists of class Ⅲ phosphatidylinositol 3-kinase (PI3K), p150 myristylated protein kinase, and beclin 1. Further elongation of the isolation membrane is mediated by two ubiquitin-like conjugation systems, Atg12-5 and microtubule-associated protein 1A/1B-light chain3 (LC3) systems. Atg12 is activated by Atg7 and transferred to Atg10, and is finally conjugated to Atg5, forming the irreversible Atg12-Atg5 conjugate[8]. The conversion of LC3 results from the free form (LC3-Ⅰ), which is transformed to a lipid-conjugated membrane-bound form (LC3-Ⅱ). Accumulation of LC3-Ⅱ and its localization to vesicular structures are commonly used as markers of autophagy.

Baseline levels of autophagy contribute to maintenance of cellular homeostasis through elimination of old or damaged organelles, as well as the turnover of long-lived proteins. Autophagy is frequently activated in response to adverse stress, and has been shown to be involved in many physiological and pathological processes. In starvation conditions, enhanced autophagy provides stressed cells with metabolic intermediates to meet their bioenergetic demands[9]. Moreover, autophagy can be dramatically augmented as a protective and survival mechanism in response to numerous conditions of extracellular or intracellular stress, including hypoxia, radiotherapy and chemotherapy[10]. On the other hand, autophagy does not always promote cell survival; it can be a mechanism of cell death under certain circumstances. For example, under experimental conditions in which apoptotic pathways are blocked, or in response to treatments that specifically trigger caspase-independent autophagy, autophagy can play a pro-death role, causing autophagic cell death[11-13].

Increasing evidence has indicated that ER stress is also a potent trigger of autophagy; another mechanism for removing unfolded proteins that cannot be eliminated by ubiquitin/proteasome system, thus mitigating ER stress and protecting against cell death. It has been reported that ER stress leads to upregulation of the transcription of genes related to autophagy induction, including *ATG8*, *ATG14* and *Vacuolar hydrolases aminopeptidase1* (*APE1*)[14]. In mammalian cells, ER stress has been shown to facilitate the formation of autophagosomes, and induction of autophagy allows removal of toxic misfolded proteins to favor survival of the stressed cells[15-17]. Another function of autophagy during ER stress is degradation of the damaged ER itself. However, autophagy induced by the same chemicals may not confer protection in normal non-transformed cells. For example, the autophagy induced by chemicals, such as A23187, tunicamycin, thapsigargin and brefeldin A protects against cell death in colon and prostate cancer cells, but contributes to cell death in normal cells[18]. ER-stress-induced autophagy is important for clearing polyubiquitinated protein aggregates and for reducing cellular vacuolization in HCT116 colon cancer cells and DU145 prostate cancer cells, thus mitigating ER stress and protecting against cell death. In contrast, autophagy induced by the same chemicals does not confer protection in a normal human colon cell line and in the non-transformed murine embryonic fibroblasts (MEFs) but rather contributes to cell death. Thus, the impact of autophagy on cell survival during ER stress is probably contingent on the status of the cells, which could be explored for tumor-specific therapy.

There is also evidence that autophagy is invoked as a means of killing cells when ER stress is implacable[18,19]. The signaling pathways responsible for autophagy induction and its cellular consequences appear to vary with cell types and the stimuli. A better understanding of the signaling pathways controlling autophagy and cellular fate in response to ER stress will hopefully open new possibilities for the treatment of the numerous diseases associated with ER stress.

CYTOPROTECTIVE AUTOPHAGY INDUCED BY ER STRESS

Initiation of autophagy has been shown to exert protective effects in yeast and mammalian cells in response to ER stress. When the amount of unfolded or misfolded

proteins exceeds the capacity of the proteasome-mediated degradation system, autophagy is triggered to remove these proteins. The observations in yeast show that ER-stress-induced autophagy counterbalances ER expansion, removes aggregated proteins from the ER, and plays a cytoprotective role in the case of intense and persistent stress[20,21]. Similarly, autophagy can also act as an ER-associated degradation system in mammalian cells, and it plays a fundamental role in preventing toxic accumulation of disease-associated mutant proteins in the ER. A mutant form of a type-Ⅱ transmembrane protein dysferlin, a causative agent of human muscle dystrophy, has recently been shown to accumulate and form aggregates in the ER and eventually lead to apoptotic cell death. Inhibition of functional autophagy in Atg5-deficient MEFs further stimulates the aggregation of mutant dysferlin, whereas enhanced autophagy in the rapamycin (mTOR inhibitor)-treated cells reduces accumulation of the mutant protein in the ER[22]. Likewise, ER aggregates of mutant α1-antitrypsin Z, which is associated with the development of chronic liver injury and hepatocellular carcinoma, induce autophagy-mediated removal of the aggregated proteins[16]. These studies did not directly assess the effect of autophagy on cell survival, but as the protein aggregates in the ER are the probable cause of cell death, autophagy capable of degrading them is envisaged to be cytoprotective. Similarly, experimental models for diseases caused by protein aggregates in the cytosol suggest that ER-stress-induced autophagy enhances removal of aggregates and enhances cell survival[23]. In addition, it has been shown that ER itself is the major autophagosomal cargo during ER stress, which suggests that the pro-survival effect of autophagy in this model system could be due to increased removal of unfolded proteins[20].

Autophagy can be protective against ER stress in several circumstances including cancer progression. Autophagy protects colon and prostate cells from ER stress and cell death induced by A23187, tunicamycin, thapsigargin and brefeldin A[18]. Treatment of neuroblastoma SK-N-SH cells with ER stressors, tunicamycin and thapsigargin, induces formation of autophagosomes, and Atg5-deficient cells and 3-methyladenine (3-MA)-treated cells demonstrate increased vulnerability to ER stress, as well as more rapid activation of cascape-3, as compared with the non-transfected and non-treated cells when subjected to ER stress[24]. Upon exposure of HeLa cells to HIV-1 Tat-induced autophagy, suppression of autophagy by 3-MA or knockdown of Atg5 significantly increases cell death, indicating that autophagy protects against cell death during ER stress[25]. These results indicate that autophagy plays pivotal roles in protecting against cell death induced by ER stress.

AUTOPHAGY AS A CELL DEATH MECHANISM IN APOPTOSIS-DEFICIENT CELLS DURING ER STRESS

ER stress can cause necrotic cell death in *bak*$^{-/-}$*bax*$^{-/-}$ cells that are defective in apoptosis, and ER-stress-induced necrosis has been known to be associated with autophagy[19,26]. Autophagy can be induced to similar levels in the wild-type and *bak*$^{-/-}$*bax*$^{-/-}$ cells in response to ER stress, but the resulting outcome of this response appears to be different. Inhibition of autophagy by 3-MA or by silencing of Atg5 using shRNA significantly enhances the viability of *bak*$^{-/-}$*bax*$^{-/-}$ cells when ER stress is present, indicating that autophagy can enhance cell death in the *bak*$^{-/-}$*bax*$^{-/-}$ cells. In contrast, 3-MA enhances ER-stress-induced cell death in apoptosis-competent wild-type cells[19]. These findings suggest that autophagy may have opposite effects in determining cell fate in response to ER stress in apoptosis-competent cells in which autophagy serves as a survival mechanism, and in apoptosis-deficient cells that utilize autophagy as a means to promote non-apoptotic cell death. This may be because, in the ER-stressed cells in which excessive stress fails to induce apoptosis, the stress status keeps escalating to a point where autophagy is massively induced, leading to subsequent cellular damage and necrosis. Therefore, autophagy may act as a death mechanism that substitutes for deficient apoptosis under ER stress *via* crosstalk with necrosis.

Autophagy can play differential role in cancer and non-transformed cells. For instance, disturbing ER homeostasis and/or functions by certain chemicals can elicit autophagy in primary colon cells, and suppression of autophagy by 3-MA reduces cell death. Nevertheless, 3-MA or depletion of beclin1 in colon carcinoma cells sensitizes tumor cells to the same treatments. In addition, suppression of autophagy induced by the same chemicals in the immortalized but non-transformed MEFs by deletion of Atg5 also reduces cell death, indicating that non-transformed cells may be especially sensitive to ER-stress-induced autophagy[18]. These observations suggest that autophagy can contribute to ER-stress-induced cell death in different scenarios, which may be dependent on cellular status under given stimulation. The differential role of autophagy in promoting survival of cancer cells or death of non-transformed cells might be related to the level at which ER stress is compensated.

SIGNALING PATHWAYS INVOLVED IN AUTOPHAGY DURING ER STRESS

Although autophagy is known to be associated with ER stress, the precise molecular mechanisms by which autophagy is activated under ER stress is not yet fully elucidated. PERK, IRE1 and increased $[Ca^{2+}]_i$ have been implicated as mediators of ER-stress-induced autophagy in mammalian cells, as depicted in Figure 1.

eIF2α is phosphorylated in response to various stresses, including starvation, viral infection and ER stress. The relationship between autophagy and eIF2α phosphorylation has been shown during starvation-induced autophagy in *Saccharomyces cerevisiae* and during starvation- and virus-infection-induced autophagy in mammalian cells[27]. Thus, it is possible that various stressful conditions that activate

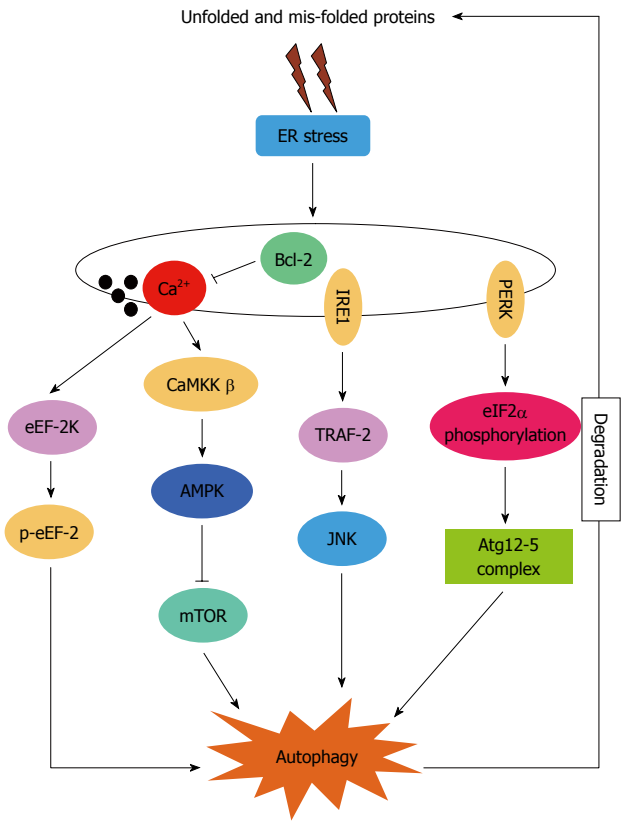

Figure 1 Hypothetical signaling pathways involved in endoplasmic reticulum stress-induced autophagy. ER: Endoplasmic reticulum; Bcl-2: B-cell lymphoma/leukemia 2; IRE1: Inositol requiring enzyme 1; eEF: Eukaryotic elongation factor; eIF: Eukaryotic initiation factor; JNK: c-Jun N-terminal kinase.

eIF2α kinases, including ER stress, may have an ability to induce autophagy in mammalian cells. Consistent with this hypothesis, the PERK-eIF2α signaling pathway has been reported to link ER stress to autophagy. A novel mutant form of a type-Ⅱ transmembrane protein dysferlin aggregates and accumulates in the ER and induces eIF2α phosphorylation and LC3 conversion. Inhibition of autophagy by depletion of Atg5 inhibits degradation of mutant dysferlin. Furthermore, dephosphorylation of eIF2α also stimulates aggregation of mutant dysferlin in the ER, suggesting that ER-stress-induced eIF2α phosphorylation may regulate autolysosome formation. Rapamycin, which induces eIF2α phosphorylation-mediated LC3 conversion, inhibits mutant dysferlin aggregation in the ER[22]. These results indicate that mutant dysferlin aggregated on the ER membrane stimulates autophagosome formation *via* activating ER-stress-induced eIF2α phosphorylation.

Kouroku *et al*[23] have reported that ER stress caused by ectopic expression of polyQ72 upregulates Atg12 expression and induces autophagy, as demonstrated by an increase in conversion of LC3-Ⅰ to LC3-Ⅱ and an increase in LC3-postive vesicles in mouse embryonic carcinoma cells and MEFs. The polyQ72-induced LC3 conversion is inhibited in cells containing the eIF2α A/A mutation and dominant negative-PERK, strongly suggesting that the PERK/eIF2α pathway, an ER stress response signal, plays an essential role in polyQ72-induced Atg12 upregulation and LC3 conversion. However, the molecular mechanism by which eIF2α phosphorylation regulates LC3 conversion remains unclear. Atg12, a component of Atg5-Atg12-Atg16 complex, as well as CHOP mRNA, are selectively upregulated by polyQ72 *via* eIF2α phosphorylation. Thus, one possible explanation is that the eIF2α phosphorylation-dependent selective translation of transcription factors increases the expression of Atg12, resulting in the formation of Atg5-Atg12-Atg16 complex, followed by conversion of LC3-Ⅰ to LC3-Ⅱ[23].

Contradictory to the above, some studies have shown that IRE1 is crucial for autophagosome formation and LC3-Ⅱ conversion after treatment with ER stressors. Imaizumi and co-workers have suggested that IRE1, rather than PERK, links UPR to autophagy[24]. Using MEFs deficient in IRE1α or ATF6 and embryonic stem cells deficient in PERK, they have demonstrated that accumulation of LC3-positive vesicles triggered by tunicamycin or thapsigargin fully depends on IRE1, but not PERK or ATF6. Thapsigargin-induced accumulation of LC3-positive vesicles is also completely inhibited in MEFs deficient in tumor necrosis factor receptor-associated factor (TRAF)-2, a cytosolic adaptor molecule that links active IRE1 to the activation of c-Jun N-terminal kinase (JNK). Additionally, a pharmacological inhibitor of JNK, SP600125, effectively inhibits the LC3 translocation in this model system, suggesting that IRE1-TRAF2-JNK pathway is essential for induction of autophagy in MEFs challenged with ER stressors. Yorimitsu *et al*[28] have reported that the Ire1-Hac1 signaling pathway is required for induction of autophagy. They have examined autophagy under ER stress conditions in the absence of Ire1 or Hac1, and have found that, in both *ire1*D$^{(-/-)}$ and *hac1*D$^{(-/-)}$ cells, ER-stress-induced autophagy was blocked. Starvation-induced autophagy was not affected in these cells. These observations suggest that under ER stress, the Ire-Hac1 signaling pathway is involved in autophagy induction; probably through the UPR.

The release of Ca^{2+} can activate various kinases and proteases that are possibly involved in the autophagy pathway. Thapsigargin increases $[Ca^{2+}]_C$ and induces autophagy, as measured by LC3 translocation, electron microscopy and degradation rate of long-lived proteins, and this is effectively inhibited by Ca^{2+} chelators[29]. The same study has further demonstrated that Ca^{2+}-mediated autophagy is dependent on the calmodulin-dependent protein kinase kinase-β/AMP-activated protein kinase pathway that ultimately leads to the inhibition of mTORC1, as demonstrated by decreased phosphorylation of the mTORC1 substrate p70S6K1.

Eukaryotic elongation factor (eEF)-2 is a 93-kDa monomeric guanine nucleotide-binding protein and is an essential mediator of the ribosomal elongation step during mRNA translation. eEF-2 promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome, and is an essential regulatory factor for protein synthesis. The phosphorylation

of eEF-2 on Thr56 by eEF-2 kinase is known to inhibit its translational function, by reducing its affinity for ribosomes[30]. It is known that eIF2α phosphorylation is required for phosphorylation of eEF-2 during nutrient starvation. eEF-2K is also required for activation of autophagy caused by various stresses, including ER stress[31], nutrient depletion[32], and Akt inhibition[33], suggesting that phosphorylation of eEF-2 serves as an integrator of various cell stresses for autophagy signaling. However, PERK and the phosphorylation of eIF2α are dispensable for eEF-2 phosphorylation during ER stress, indicating that eEF-2 phosphorylation can be triggered by multiple signaling pathways, including the PERK/eIF2α pathway. The phosphorylation of eEF-2 by tunicamycin or thapsigargin treatment is significantly inhibited in the presence of the Ca^{2+} chelator BAPTA-AM, indicating that activation of eEF-2 kinase relies on Ca^{2+} flux during ER stress. Thus, phosphorylation of eEF-2 may be a common mediator of autophagy during starvation or ER stress. These results suggest that eEF-2 kinase plays an important regulatory role in mediating autophagy in response to multiple stress stimuli, and can be activated in an eIF2α-dependent or -independent manner.

B-cell lymphoma/leukemia 2 (Bcl-2) is an anti-apoptotic protein located at mitochondrial, ER and nuclear membranes, and to a lesser extent in the cytoplasm. Accumulating evidence suggests that Bcl-2 can inhibit or activate autophagy, depending on different model systems. The opposite effects of Bcl-2 on autophagy may be attributed to its post-translational modifications or different subcellular localizations. Inhibition of autophagy by Bcl-2 is shown by the fact that it blocks autophagosome accumulation induced by starvation, vitamin D analog EB1089, ATP and Xestospogin B[18,29,34]. At least two mechanisms have been proposed for Bcl-2-mediated inhibition of autophagy: a direct interaction with beclin 1; and regulation of ER Ca^{2+} stores, possibly *via* its binding to IP3R[29,34]. Beclin 1 is a Bcl-2-interacting protein that promotes autophagosome formation when in complex with class Ⅲ PI3K and p150 myristylated kinase. Bcl-2 has been suggested to function as an autophagy brake by inhibiting the formation of this autophagy-promoting protein complex. ER-localized Bcl-2 lowers the steady-state level of Ca^{2+} in the ER and thereby reduces stimulus-induced Ca^{2+} fluxes from the ER. Thus, it may inhibit Ca^{2+}-dependent autophagy by reducing the increase in $[Ca^{2+}]_C$. This hypothesis is supported by data showing that ER-localized Bcl-2 effectively inhibits autophagy induced by Ca^{2+} mobilizing agents that depend on ER Ca^{2+} stores (EB1089and ATP)[35]. Bcl-2 at the ER may depend on beclin1 binding to decrease the amount of Ca^{2+} released from the ER following agonist stimulation. Alternatively, ER-targeted Bcl-2 may be able to inhibit autophagy by other means, depending on the signaling pathway involved in autophagy induction.

CONCLUSION

Autophagy is important for the clearance of unfolded/misfolded proteins and for relief of ER stress induced by various stresses. The current studies, as discussed above, encourage the development of autophagy-promoting therapies for diseases associated with protein aggregates in the ER or cytosol. It is known that activation of ER stress and autophagy is associated with dealing with amyloid β-peptide accumulation in the brain; the major cause of Alzheimer's disease. In cancer cells, autophagy helps to alleviate ER stress, and inhibits cell death. If this proves to be the case, combination therapies with ER stressors and autophagy inhibitors may also be useful in cancer therapy. The direct link between ER stress and autophagy has been reported for less than 1 year. Thus, many burning questions concerning the signaling pathways linking ER stress to autophagy, the mechanisms by which ER is selected as autophagic cargo, the crosstalk between ER-stress-induced autophagy and cell death pathways (apoptosis and necrosis), and the impact of autophagy in diseases associated with ER stress, remain largely unanswered. Future research will hopefully clarify these issues and pave the way for pharmacological exploitation of the signaling pathways involved in crosstalk between autophagy and apoptosis or necrosis. As the roles of autophagy can either be pro-survival or pro-death depending on context, it is conceivable that manipulating autophagy would have an impact on therapeutic outcome of various diseases. For instance, if autophagy activation contributes to resistance of cancer cells to certain therapies, use of autophagy inhibitors could be beneficial; in contrast, if induction of autophagy facilitates cell killing by cancer therapeutics, co-treatment with autophagy activators could reinforce the therapy. How to exploit autophagy as a therapeutic intervention remains an area of extensive investigation.

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TOPIC HIGHLIGHT

Hui-Ling Chiang, PhD, *Series Editor*

Mechanisms of autophagy and apoptosis: Recent developments in breast cancer cells

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Abstract

Autophagy, the pathway whereby cell components are degraded by lysosomes, is involved in the cell response to environmental stresses, such as nutrient deprivation, hypoxia or exposition to chemotherapeutic agents. Under these conditions, which are reminiscent of certain phases of tumor development, autophagy either promotes cell survival or induces cell death. This strengthens the possibility that autophagy could be an important target in cancer therapy, as has been proposed. Here, we describe the regulation of survival and death by autophagy and apoptosis, especially in cultured breast cancer cells. In particular, we discuss whether autophagy represents an apoptosis-independent process and/or if they share common pathways. We believe that understanding in detail the molecular mechanisms that underlie the relationships between autophagy and apoptosis in breast cancer cells could improve the available treatments for this disease.

Key words: Autophagy; Apoptosis; Survival; Breast cancer cells; Signaling pathways

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AUTOPHAGY

Autophagy is the process whereby organelles and other cell components are degraded by lysosomes. There are various types of autophagy, including macroautophagy, microautophagy and chaperone-mediated autophagy[1]. Macroautophagy, hereafter called autophagy, is the most important form of autophagy and involves the formation of double-membrane vacuoles, named autophagosomes, containing cytosol and organelles. Autophagosomes then fuse with endosomes and lysosomes to form autolysosomes (Figure 1), which undergo a gradual acidification, by a proton pump, and degradation, by hydrolytic enzymes, of their content[2]. Autophagosome formation is a complex mechanism in which different autophagy-related (Atg) proteins participate, including Beclin 1 and LC3 (Atg6 and Atg8 in yeast, respectively), and which also requires the cell cytoskeleton[1,3,4]. Autophagy occurs at basal levels in almost all cells, and its main function is the degradation of cell components, including long-lived proteins, protein aggregates and organelles produced in excess, aged, damaged and potentially dangerous or no longer needed[5,6]. Under starvation conditions, autophagy provides the cells

with molecules (amino acids, fatty acids, monosaccharides and nucleotides) that can be used for biosynthetic purposes. Some of these molecules can also be utilized as energy sources and the ensuing biosyntheses require energy. Therefore, it appears logical that part of them can be used to produce this energy, as has been postulated by many authors[7-10]. However, direct experimental proof for a role of autophagy in restoring the energy levels in the cell is still missing, probably because of the difficulties derived from the fact that this energy would be immediately used by the cells recovering from stress. Autophagy has also an important role in normal development, differentiation, and tissue remodeling in multicellular organisms, as well as in their adaptation to several stresses[5,11].

Regarding cancer, which is the general subject of this Topic Highlight, a tumor suppressor role for autophagy has been also proposed, removing injured mitochondria that could increase the production of reactive oxygen species (ROS) and the number of mutations in cancer cells[11].

Role of autophagy in survival and death of tumor cells in response to environmental stress

In the previous decade, several reports have suggested a role for autophagy in cell survival at different stages of tumor development and in the tumor cell response to anticancer therapy[4,11,12], and this role of autophagy has become a major research topic. Under stress conditions, like deprivation of growth factors or nutrients, hypoxia or exposition to chemotherapeutic agents, cells induce autophagy to provide biosynthetic precursors and, perhaps also (but see above), energy, or to eliminate injured cell components, thus preventing cell death[7,13,14]. Therefore, autophagy may allow cancer cells to survive under nutrient and oxygen-poor conditions, reminiscent of certain microenvironments in poorly vascularized tumors[15]. Autophagy can also contribute to cell survival by removing injured targets of ROS and proteins carrying mutations that could lead to an irreversible stage conducive to cell death[16]. Under the aggressive stress conditions experienced by tumor cells, their autophagy levels are higher than normal and, therefore, disruption of this increased autophagy by therapeutic manipulations will make difficult the adaptation of these cells to extreme environments, and contribute to cancer therapy. However, chemical inhibitors of autophagy also prevent the death of cancer cells induced by a variety of agents[17]. This opposite role of autophagy as an executioner of cell death[18-20] and, thus, playing a role as a tumor suppressor[11], could probably be explained by a persistent degradation of components essential for cell survival[4,21]. Therefore, it appears that, in addition to its conventional role in cell survival, autophagy can be also a death-promoter, in particular when the stimulus is too intense, when autophagy is extensive, or under conditions of inhibition of apoptosis. The level of autophagy that represents the point of no return leading to cell death has not been clearly defined and should be determined experimentally in each specific system. However, some authors have considered that a situation in which the total area of autophagic vacuoles is equal or greater than that of the remaining cytoplasm would irreversibly lead to cell death[20,22].

In all these cases, the conventional inhibitors of autophagy and the concentrations used by most authors to block or promote survival of cancer cells under *in vitro* conditions[13,14,18,23,24] were the following: 3-methyladenine (5-10 mmol/L), chloroquine (10 μmol/L) and bafilomycin A1 (0.1 μmol/L). To the best of our knowledge, these chemicals have not yet been used for clinical treatment of cancer, except for chloroquine, which has been used in patients with glioblastoma multiforme. Thus, in these antitumoral clinical trials, chloroquine, or its lower toxicity analog hydroxychloroquine, have been used (150 mg/d, for 12 mo) as autophagy inhibitors in combination with proapoptotic drugs, increasing, in this way, twofold the median survival of these patients[25-28].

In summary, autophagy may either promote or inhibit survival in tumor cells, and the threshold to decide between both opposite processes will depend on the extent of the cell degradation produced[29], as well as on many other factors, such as the genetic context of the cell and the nature and intensity of the stimulus needed to reduce cell survival[30].

Autophagy in the context of cell death

In recent decades, studies in the field of cell death have focused on understanding the molecular mechanisms of apoptosis (often called programmed cell death, and now also referred to as cell death type I). Apoptosis is the form of cell death in which a group of cysteinyl aspartate-specific proteases, called caspases, become activated to cleave different proteins (and the caspases themselves) that ultimately produce loss of cell function, and cell death. In apoptosis, initiator caspases (2, 8, 9 and 10) activate executioner caspases (3, 6 and 7, of which, caspase-3 is the major and most widespread effector of the process)[31,32]. The essential feature of apoptosis, which makes it different from classical necrosis, is that it is a self-directed cell destruction process through caspase activation. Hundreds of caspase substrates have been described[33] and different biochemical and morphological changes in the nucleus and cytoplasm (e.g. cell contraction, membrane blebbing, externalization of phosphatidylserine, chromatin condensation into one or more masses, DNA fragmentation, limited proteolysis of certain substrates, and heterophagic elimination of apoptotic bodies by neighboring cells) have been used to identify apoptotic cells[17,34,35]. Two well-established molecular pathways (extrinsic and intrinsic) activate caspases and trigger apoptosis. The first is the death-receptor-mediated pathway, which is activated by ligands that bind to specific receptors on the plasma membrane, such as the tumor necrosis factor receptor 1 and Fas. The other is the mitochondrial pathway, which takes place through permeabilization of these organelles, followed by the release of apoptotic molecules such as cytochrome c (which triggers the formation of larger complexes called apoptosomes), apoptosis-inducing factor (AIF), or endonuclease G[17,31-33].

In addition to canonical apoptosis and necrosis, diverse experimental evidence has shown that cells can die

through alternative pathways[36]. Thus, there is a form of cell death, whose main feature is the appearance of abundant autophagic vacuoles in the cytoplasm of dying cells, known as autophagic or type Ⅱ cell death, and several of its characteristics, based mainly on morphological criteria, have been described in recent years[20]. Type Ⅱ cell death would occur because of persistent autophagy with excessive degradation of cell components essential for survival[4,21], and it is usually accompanied by inhibition of the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3kinase/Akt/mTOR) signaling pathway[37,38], which is the main regulator of autophagy, and by increased levels of LC3-Ⅱ[1], a protein that is recruited to autophagosomes and that, under certain conditions, can be used as a reliable marker for autophagy[39,40]. However, different studies have found that some of the apoptotic cell death features cited above are also associated with an increased autophagy[18,29]. Therefore, the question is raised as to whether or not apoptosis and autophagy represent two independent processes.

In this regard, different reports indicate that autophagy can act independently of the apoptotic signaling pathways. Thus, because preservation of most cytoplasmic organelles is among the classic hallmarks of apoptosis, autophagic cell death, which comprises an extensive sequestration and degradation of mitochondria, endoplasmic reticulum (ER) and other cell components, has been considered by some authors as a different category of cell death on its own[41,42]. In addition, other evidence supports that extensive autophagy may be a caspase-independent form of cell death. For example, blockage of caspase activity prevents Bax-induced poly (ADP ribose) polymerase and DNA cleavage, but not cytosolic vacuolation and non-apoptotic cell death[43]. In the same lines of evidence, it has been shown that death-associated protein kinase proteins positively regulate membrane blebbing and autophagy, but apparently not nuclear fragmentation, and that these events occur in a caspase-independent manner[44].

However, it is also quite clear that autophagy can also coexist and crosstalk with apoptosis. Indeed, several molecules that regulate apoptosis are among the different targets of the PI3-kinase/Akt/mTOR signaling pathway[45,46] and proteins, such as Beclin 1, phosphatase and tensin homolog, apoptosis-specific protein, and the product of the steroid-inducible gene *E93* can establish interconnections between autophagy and apoptosis[29,42]. Therefore, different evidence appears to indicate that apoptosis and extensive autophagy represent two forms of cell death with independent, but also with common pathways (Figure 2). However, the molecular details of these latter relationships remain poorly known.

MOLECULAR MECHANISMS OF AUTOPHAGY AND APOPTOSIS IN BREAST CANCER CELLS

As mentioned above, autophagy can promote or inhibit tumor survival depending on many factors, such as the specific cell type with the set of mutations that it carries, the stage of tumor development, and the stimulus that induces autophagy plus the extent of the resulting autophagy. Therefore, being aware of the heterogeneity in the survival/death response, which makes it difficult to generalize the different observations, and to limit the problem, we update the data on this topic in breast cancer cells. We have chosen these cells because of the growing number of recent studies on the role of autophagy in survival and death, compared to other experimental models.

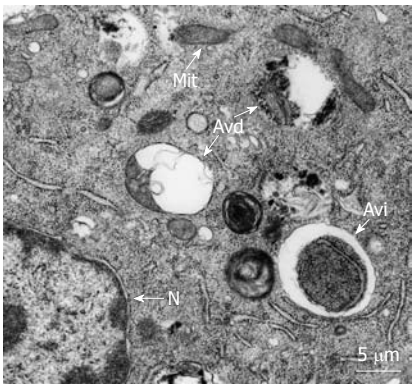

Figure 1 Morphology of autophagic vacuoles. Typical autophagic vacuoles from 3T3 mouse fibroblasts incubated in a nutrient-poor medium containing cytoplasmic material at early (Avi) and late (Avd) degradation stages. Mit: Mitochondria; N: Nucleus.

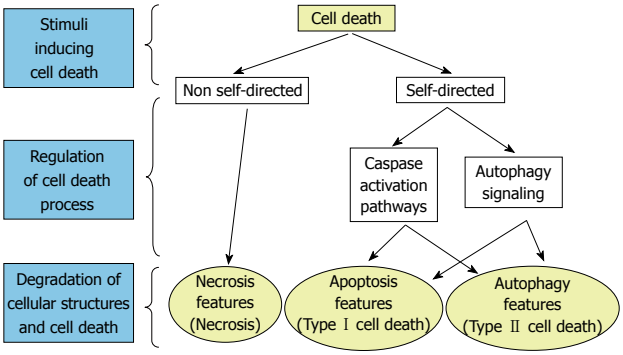

Figure 2 Main forms of cell death. Autophagy is located in the proper context in relation to classical necrosis and apoptosis. Crossing arrows indicate the existence of common links for apoptosis and autophagy. The entire process of cell death has been divided into three phases: stimulation, regulation and degradation. Note the absence of a regulation phase in necrosis.

Role of autophagy in survival and death of breast cancer cells in response to environmental stress

Studies on autophagy in breast cancer cells, mainly in MCF-7 cells, indicate that, in chemotherapeutic treatments, induction of autophagy plays a protective role in the resistance to apoptosis induced by anticancer drugs, such as the inhibitor of DNA topoisomerase I camptothecin[29], epirubicin[47], which intercalates DNA strands, different ligands that stimulate the antiestrogen binding site (AEBS), including tamoxifen[48,49], or 4-hydroxytamoxifen, an active metabolite of tamoxifen that binds to the es-

Table 1 Agents inducing anticancer mechanisms in cultured breast cancer cells

Agent	Model	Anticancer mechanism	Citation
Camptothecin	MCF-7	Apoptosis ↑	[29]
Epirubicin	MCF-7	Apoptosis ↑	[47]
Tamoxifen	MCF-7	Apoptosis ↑	[48,49]
4-hydroxytamoxifen	MCF-7, T-47D	Apoptosis ↑	[50,51]
Lucanthone	MDA-MB-231	Apoptosis ↑, autophagy ↓	[25,53]
Chloroquine	Breast cancer carcinoma[1]	Apoptosis ↑, autophagy ↓	[69]
Photodynamic therapy	MCF-7	Autophagy ↑	[56]
Tunicamycin	MCF-7	Autophagy ↑	[59]

[1]*Ex vivo* model.

trogen receptor α[50,51]. Consistent with this idea, treatment of estrogen-receptor-positive breast cancer cells with the antiestrogen tamoxifen, combined with histone deacetylase inhibition, maintains a subpopulation of cells with an elevated autophagy and a remarkable resistance to apoptosis. These apoptosis-resistant cells only become apoptotic after inhibition of autophagy[52]. Also, and in the same line of evidence, the anticancer properties of lucanthone have been recently related to its ability to induce apoptosis and inhibit autophagy in breast cancer cell lines[53]. Further indications for a promoting effect on breast malignant cell development by autophagy are provided by recent reports showing that the tumor suppressor BRCA1 (breast cancer type 1 susceptibility) negatively regulates autophagy in MDA-MB-231[54] and in MCF-7[55] breast cancer cells. Thus, it could be that mutations in the *BRCA1* gene or reduced expression of the encoded protein facilitate tumor development by preventing apoptosis through autophagy activation. Nevertheless, a death-promoting effect has also been reported for autophagy; for example, in MCF-7 cells subjected to oxidative damage by photodynamic therapy[56] or in MCF-7 cells overexpressing Bcl-2 in the presence of the antineoplastic factor brevinin-2R[57]. Table 1 shows the specific anticancer effects on apoptosis and/or autophagy of various agents tested under *in vitro* conditions in breast cancer cells.

In conclusion, in breast cancer cell lines, autophagy mainly facilitates their survival and adaptation to adverse environments, whereas apoptosis has the opposite effect, and the final outcome, in terms of survival or death of the cells, will depend on many factors. Therefore, it appears that, at least in breast cancer cells, both apoptosis induction and autophagy inhibition have positive therapeutic implications depending on context.

Functional links of autophagy and apoptosis in cultured breast cancer cells

In breast cancer MCF-7 cells, camptothecin induces both apoptosis, demonstrated by deficient (sub-G1) DNA content and by chromatin condensation, and autophagy, demonstrated by increased levels of Beclin 1 and autophagosomes[58]. Also, in various breast cancer cells, sterol accumulation promoted by binding of various ligands, such as tamoxifen, to microsomal AEBS, induces both apoptosis and autophagy[48,49]. However, other treatments have opposite effects in both processes (Table 1). For example, in MDA-MB-231 breast cancer cells, lucanthone induces apoptosis and inhibits autophagy[25]. This experimental evidence suggests the existence of common links between apoptosis and autophagy in breast cancer cells. However, the door to the molecular mechanisms that link apoptosis and autophagy in breast cancer cells has only recently begun to open, and current knowledge is discussed below.

Thus, different proteins that belong to the mitochondrial pathway of apoptosis have also been shown to crosstalk with Atg proteins and to regulate autophagy in cultured breast cancer cells. For example, in MCF-7 cells, which lack caspase-3, expression of an ectopic caspase-3 reduces the enhanced autophagy produced by tunicamycin (an inductor of ER stress) or/and by radiation[59]. This effect is accompanied by a decrease in the levels of phosphorylated eukaryotic initiation factor 2α, which at the same time increases protein synthesis[59]. Therefore, caspase-3 may be a switch between type I and Ⅱ cell death[17,60]. In these same cells, activation of another apoptosis promoter, protein Bid, also affects apoptosis and autophagy in opposite directions, because it not only stimulates apoptosis but also reduces autophagy by inhibition of Beclin 1[58]. In contrast, and also in MCF-7 cells, the antiapoptotic protein Bcl-2 regulates both processes in the same direction, because it negatively regulates the levels of three Atg proteins (Beclin 1, Atg5 and LC3-Ⅱ), thus inhibiting autophagy[61]. Recently, a gene network signaling model has also indicated a central role for Bcl-2 and Beclin 1 in the apoptotic and autophagic responses to endocrine therapies in breast cancer cells, and has identified nuclear factor κB, interferon regulatory factor-1, and the X-box binding protein-1 as new key proteins that regulate Bcl-2 and Beclin 1 in these responses[62].

Unlike the apoptotic regulation of autophagy in breast cancer cells, a possible control of apoptosis by autophagy remains to be investigated in detail. However, it is known in other cell types that the PI3-kinase/Akt/mTOR signaling pathway, which has an inhibitory effect on autophagy, can interact with proteins that regulate apoptosis[45,46]. Moreover, it has been speculated that the selective removal of damaged mitochondria generating ROS by autophagy (mitophagy) could inhibit the mitochondrial pathway of apoptosis[6,63,64]. Furthermore, lysosomal cathepsins can establish a link between apoptosis and autophagy, because they are released from lysosomes into the cytosol in response to death stimuli, and induce apoptosis[65]. More specifically, it has been described in other cell lines that cathepsin D activates the proapoptotic protein Bax, which triggers the release of AIF from mitochondria[66], and that papain-like lysosomal cathepsins are able to cleave the proapoptotic protein Bid[67]. Also in MCF-7 breast cancer cells, papain-like cysteine cathepsins, proba-

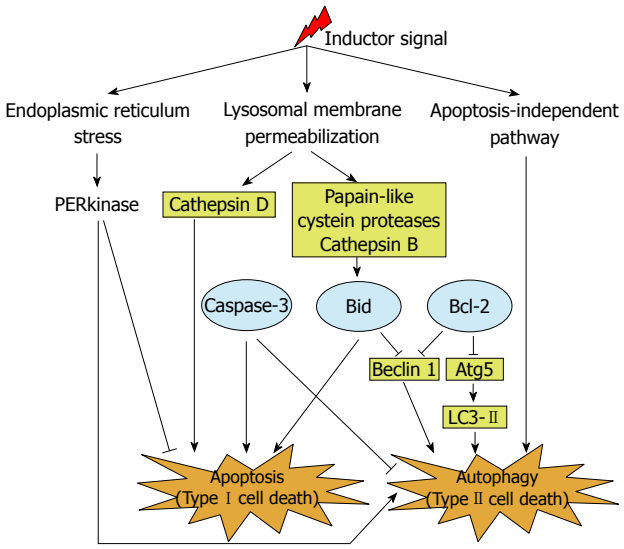

Figure 3 Model of regulation of autophagy and apoptosis in breast cancer cells. Different molecules described to act as a link between apoptosis and autophagy are shown (see text for details). Ellipses indicate classical regulators of apoptosis. Moreover, organelles, such as mitochondria, endoplasmic reticulum and lysosomes, appear to be involved in this regulation. Arrow-headed lines and bar-headed lines indicate activation and inhibition, respectively, of their corresponding targets.

bly including cathepsin B[58], activate Bid, which promotes apoptosis and reduces autophagy. A further example of a lysosomal cathepsin regulating apoptosis is provided by MDA-MB-231 breast cancer cells, in which lucanthone inhibits autophagy, probably by affecting lysosomal acidification, and induces a cathepsin-D-mediated apoptosis. This apoptosis probably occurs by lysosomal membrane permeabilization, subsequently releasing cathepsin D into the cytosol, which cleaves caspases[25,53].

In addition to mitochondria and lysosomes, the ER has also been shown to be involved in the regulation of autophagy and apoptosis. Thus, in MCF-7 cells, the ER transmembrane protein kinase-like ER kinase (PERK) increases autophagy and reduces the fraction of cells that survive radiation and/or a treatment with tunicamycin, and this PERK-controlled autophagy can be inhibited by caspase-3[59].

Thus, the above-mentioned examples support a molecular link between autophagy and apoptosis. In contrast, in breast adenocarcinoma MCF-7 cells overexpressing Bcl-2, the antineoplastic factor brevinin-2R leads to mitochondrial dysfunction (demonstrated by a reduction in mitochondrial membrane potential and in cellular ATP levels, and by an increase of ROS levels), autophagosome formation and cell death. These effects occur without involving apoptotic effectors (such as caspase activation and the mitochondrial release of the AIF or of endonuclease G)[57]. Thus, it appears that autophagic cell death can also occur independently of apoptosis. All these molecular mechanisms are summarized in Figure 3.

Although this Topic Highlight is focused on breast cancer cells *in vitro*, and limited information is available *in vivo*, we briefly summarize the most relevant information available under these last conditions. In a breast tumor xenograft model, Bcl-2 reduces autophagy by inhibition of Beclin 1, as it also occurs *in vitro*[68]. Moreover, samples from patients with breast ductal carcinoma and their corresponding mouse xenografts, show an increase in many autophagic markers, and this autophagy is necessary for the *ex vivo* survival of all these samples, as shown with 50 μmol/L chloroquine[69]. This observation is again in agreement with the survival function for autophagy observed *in vitro*. Interestingly, as we discussed above, the use of chloroquine in clinical trials has increased the survival of glioblastoma patients[25-28]. Therefore, all these data support that inhibition of autophagy offers a potential therapy in breast cancer.

In summary, several lines of evidence under *in vitro* conditions indicate that, in breast cancer cells, although apoptosis and autophagy can coexist as independent pathways, they are also interconnected processes. Molecular links are represented by classic apoptosis-regulator proteins (caspase-3, Bid and Bcl-2), which inhibit autophagy by acting on Atg proteins. Upstream of these regulators of apoptosis are cytosol-released lysosomal cathepsins, which induce apoptosis by activating proapoptotic proteins. In addition, new candidates to interact with these proteins that link apoptosis and autophagy are now emerging, as illustrated by the above-mentioned studies with a gene network signaling model, and elucidation of their specific function could contribute to understand further this complex mechanism.

CONCLUSION

Autophagy is a physiological process of lysosomal degradation that, in response to environmental stresses, may either promote cell survival or death depending on many factors. In addition to canonical apoptosis (type I cell death) and necrosis, extensive autophagy represents an alternative form of cell death (type Ⅱ). In breast cancer cells, autophagy and apoptosis share some common proteins from their signaling routes. Thus, classical regulators of apoptosis, such as Bid, Bcl-2 and caspases, appear to crosstalk with Atg proteins and, in consequence, regulate autophagy. Moreover, lysosomal cathepsins provide an important link between both processes, by acting on target proteins of the apoptotic signaling pathways. However, autophagy in breast cancer cells can also be an apoptosis-independent process. Therefore, the relationships between autophagy and apoptosis are quite complex, but we predict that a better understanding of the underlying molecular mechanisms could contribute in the near future to anticancer therapy.

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Events Calendar 2011

January 19-20,
BioBusiness
London, United Kingdom

January 27-28
Predictive Human Toxicity and ADME/Tox Studies 2011
Brussels, Belgium

January 29-February 2
LabAutomation 2011
Palm Springs, United States

February 1-2
2011 Pharma Market Research Conference
Parsippany, United States

February 6-8
5th Drug Discovery for Neurodegeneration
San Diego, United States

February 7-10
3rd International Conference and Exhibition on Drug Discovery and Therapy
Dubai, United Arab Emirates

February 13-16
Natural Products Conference 2011
Sharm el Sheikh, Egypt

February 14-17
Therapeutic Approaches to Neurodegeneration - Age Modifiers, Proteostasis, and Stem Cells
Nassau, Bahamas

February 16-19
Electrochemistry Conference 2011
Sharm el Sheikh, Egypt

February 21-23
World Antibody Drug Conjugate Summit Frankfurt, Germany

February 22-24
2011 International Conference on Bioinformatics and Computational Biology III ROUND
Haikou, China

February 22-25
Medicinal Chemistry Conference 2011
Sharm el Sheikh, Egypt

February 23-25
International Conference on Bioscience, Biotechnology, and Biochemistry
Penang, Malaysia

February 26-28
2011 International Conference on Bioscience, Biochemistry and Bioinformatics
Sentaosa, Singapore

March 4
Discussion Workshop: Perfecting the ELISPOT - a time for answers
London, United Kingdom

March 4-11
Inorganic Reaction Mechanisms
Gordon Research Conferences
Galveston, United States

March 7-8
Fragments 2011 - Third RSC-BMCS Fragment-based Drug Discovery meeting
Stevenage, United Kingdom

March 9-13
10th International Conference on Alzheimers and Parkinsons Diseases
Barcelona, Spain

March 13-18
Pittcon 2011
Atlanta, United States

March 17-20
EMBO|EMBL Symposia: Seeing is Believing - Imaging the Processes of Life
Heidelberg, Germany

March 20-22
The molecular biology of inflammatory bowel diseases
Durham, United Kingdom

March 21-23
World Congress on Biotechnology
Hyderabad, India

March 23-25
BIT's 4th Annual Protein and Peptide Conference
Beijing, China

March 25-27
2011 3rd International Conference on Bioinformatics and Biomedical Technology 3rd round call for paper
Sanya, China

March 27-April 2
EMBO Practical Course - Methods in Chemical Biology
Heidelberg, Germany

April 6-8
Faraday Discussion 150: Frontiers in Spectroscopy
Basel, United States

April 6-8
Membrane Proteins: Structure and Function
Oxford, United Kingdom

April 11-12
7th SCI-RSC symposium on Proteinase Inhibitor Design
Basel, United States

April 11-14
First EuCheMS Inorganic Chemistry Conference (EICC-1)
Manchester, United Kingdom

April 18-19
Analysis of free radicals, radical modifications and redox signalling
Birmingham, United Kingdom

April 20-21
BioFine Europe Exhibition 2011
Cambridge, United Kingdom

May 1-6
46th EUCHEM Conference on Stereochemistry
Brunnen, United States

June 1-5
EMBO Conference Series - Chromatin and Epigenetics
Heidelberg, Germany

June 15-17
Spectroscopy - Detective in Science
Rostock, Germany

June 15-18
3rd International Symposium on Metallomics
Münster, Germany

July 11-13
Ubiquitin Conference
Philadelphia, United States

July 17-22
Charge Transfer in Biosystems - ESF-LFUI Conference
Obergurgl, United States

July 18-20
2nd International Congress on Analytical Proteomics
Ourense, United States

August 3-4
From beads on a string to the pearls of regulation: the structure and dynamics of chromatin
Cambridge, United Kingdom

August 7-12
15th International Conference on Biological Inorganic Chemistry (ICBIC 15)
Vancouver, United States

August 28-September 2
Microscopy Conference 2011
Kiel, Germany

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INSTRUCTIONS TO AUTHORS

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The columns in the issues of *WJBC* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Articles: To report innovative and original findings in biochemistry and molecular biology; (9) Brief Articles: To briefly report the novel and innovative findings in biochemistry and molecular biology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJBC*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of biochemistry and molecular biology; and (13) Guidelines: To introduce Consensuses and Guidelines reached by international and national academic authorities worldwide on the research in biochemistry and molecular biology.

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the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

Journals

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics
Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
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