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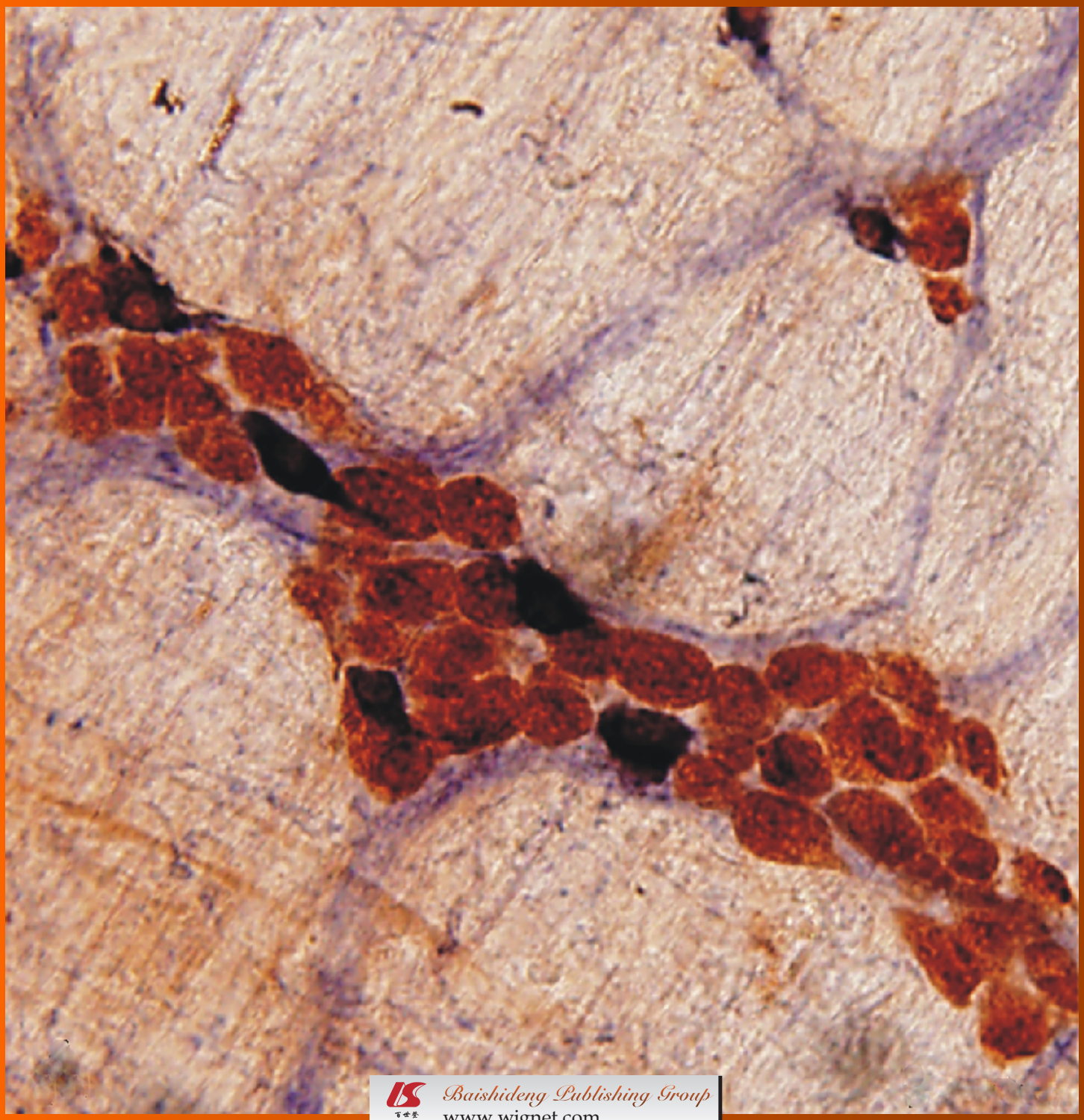

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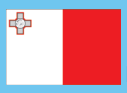

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REVIEW

Diabetes-related alterations in the enteric nervous system and its microenvironment

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Abstract

Gastric intestinal symptoms common among diabetic patients are often caused by intestinal motility abnormalities related to enteric neuropathy. It has recently been demonstrated that the nitrergic subpopulation of myenteric neurons are especially susceptible to the development of diabetic neuropathy. Additionally, different susceptibility of nitrergic neurons located in different intestinal segments to diabetic damage and their different levels of responsiveness to insulin treatment have been revealed. These findings indicate the importance of the neuronal microenvironment in the pathogenesis of diabetic nitrergic neuropathy. The main focus of this review therefore was to summarize recent advances related to the diabetes-related selective nitrergic neuropathy and associated motility disturbances. Special attention was given to the findings on capillary endothelium and enteric glial cells. Growing evidence indicates that capillary endothelium adjacent to the myenteric ganglia and enteric glial cells surrounding them are determinative in establishing the ganglionic microenvironment. Additionally, recent advances in the development of new strategies to improve glycemic control in type 1 and type 2 diabetes mellitus are also considered in this review. Finally, looking to the future, the recent and promising results of metagenomics for the characterization of the gut microbiome in health and disease such as diabetes are highlighted.

Key words: Diabetes; Enteric neurons; Insulin; Enteric neuropathy; Nitrergic neurons

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INTRODUCTION

The gastrointestinal (GI) tract accomplishes a remarkable variety of functions, such as transport of luminal content, secretion and absorption of ions, water and nutrients, defence against pathogens and elimination of waste and/or noxious substances. Digestive functions are regulated by a complex neural network, known as the enteric nervous system (ENS), endowed in the gut wall and extending throughout its length from the esophagus to the internal anal sphincter[1-11]. The ENS derives from the neural crest[4-11] and consists of neurons distributed in two ganglionated plexuses, myenteric (Auerbach's) and sub-

mucosal (Meissner's), located within the gut wall. Enteric neurons can be identified according to their function, location, neurochemistry, shape, projections, quantitative properties and connections. After intensive research from several laboratories over the past two decades, a full description of all functional classes of enteric neurons has been recently achieved in the guinea pig and the mouse intestine[11-17].

GI motility disorders, such as vomiting, constipation, diarrhea and fecal incontinence, often accompany type-1 diabetes, both in patients and in animal models[18-20]. During the past decade, a growing amount of evidence has indicated[21-22] that the nitrergic subpopulation of myenteric neurons are the main points of attack of diabetic insults in the gut. Additionally, different susceptibility of nitrergic neurons located in different intestinal segments to diabetic damage and their different levels of responsiveness to insulin treatment have been revealed[22]. These findings implied that the development of diabetic nitrergic neuropathy is more complicated than suggested earlier[21] and that it differs from segment to segment along the GI tract. These findings initiated the investigation of the capillary endothelium within the gut wall and the glial cells surrounding enteric ganglia. The most recent evidence accumulating from these studies[23-25] prove that these cells play a determinative role creating the proper microenvironment for the ENS. Therefore, knowing the diabetes-related changes of these cells is important, not only with respect to pathogenesis, but also to therapeutic points.

FUNCTIONAL AND NEUROCHEMICAL CLASSES OF ENTERIC NEURONS

In functional terms, intrinsic primary afferent neurons are determinative in the generation of intrinsic GI reflexes and also participate in the reflexes between the gut tube and accessory glands like the pancreas and liver[26-28].

There are five main types of enteric motor neuron: excitatory and inhibitory muscle motor neurons, motor neurons innervating endocrine cells, secretomotor/vasodilator neurons and simple secretomotor neurons[12]. Excitatory muscle motor neurons release acetylcholine and tachykinins, while inhibitory muscle motor neurons release nitric oxide (NO), adenosine triphosphate and vasoactive intestinal peptide. Besides muscle motor neurons, one type of orally directed (ascending) and three types of anally directed (descending) interneurons have been identified in the small intestine of the guinea pig[12].

All classes of enteric neurons are integrated in a continuous overlapping network along the GI tract. Small rings of circular muscle can contract independently; these rings and the associated enteric neurons can be regarded as functional modules. The spatiotemporal coordination of these interconnected modules is the determining factor for the generation of the rich repertoire of motor patterns[15,17].

ENS is also referred to as the "second brain" because of its capability to function in the absence of nerve inputs from the central nervous system[29]. However, extrinsic nerve pathways contribute to the regulatory mechanisms underlying gut functions[2,30-32].

NON-NEURONAL CELLS IN THE ENS

Enteric glial cells (EGCs) represent an extensive but relatively poorly described cell population within the GI tract. The EGCs network has trophic and protective functions toward enteric neurons and is fully implicated in the integration and the modulation of neuronal activities[33-35]. In addition, EGCs within the ENS have a significant role in forming a diffusion barrier around the capillaries surrounding ganglia similar to that of blood-brain barrier[3,15,36-38].

Interstitial cells of Cajal (ICCs) are also related to the ENS and are electrically coupled to the smooth muscle cells. These pacemaker cells generate spontaneous electrical slow waves and mediate inputs from motor neurons[3,39-42]. ICCs are associated with afferent innervation and peristalsis of the stomach, suggestive of a key role in the pathophysiology of gastroparesis[43-47].

NITRERGIC NEURONS

Nerve cells where transmission is mediated by NO are called nitrergic neurons[48-50]. In many organs of the urogenital, GI and cardiovascular systems, nitrergic neurotransmission plays a significant role as a major non-adrenergic non-cholinergic (NANC) neurotransmitter[51]. Nitrergic neurons in the myenteric plexus (MP) are inhibitory muscle motor neurons and descending interneurons[12,52,53].

There have already been numerous investigations of the density and spatial distribution of nitrergic myenteric neurons[54-56]. In the MP of different mammalian species, nitric oxide synthase (NOS)-immunoreactive neurons constitutes approximately 25%-40% of the total myenteric neurons[14,56,57]. It is well established that within the ENS the neuronal NOS (nNOS) corresponds to nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d); therefore, NADPH-d histochemistry is used to label nitrergic enteric neurons (Figure 1).

Various reports have described the plastic remodeling of the nitrergic neurons during development[55,58,59], aging[60] and pathological conditions[22,61]. Several studies have suggested that nitrergic myenteric neurons are especially susceptible to the development of neuropathy in digestive tract diseases, like diabetes[22,62-65], chronic ethanol consumption[66-68] and inflammation[69].

NITRERGIC ENTERIC NEUROPATHY IN DIABETES

Diabetes-related abnormalities in the ENS were reviewed in 2007[70]. Recent studies on the ENS in diabetes are summarized in Table 1.

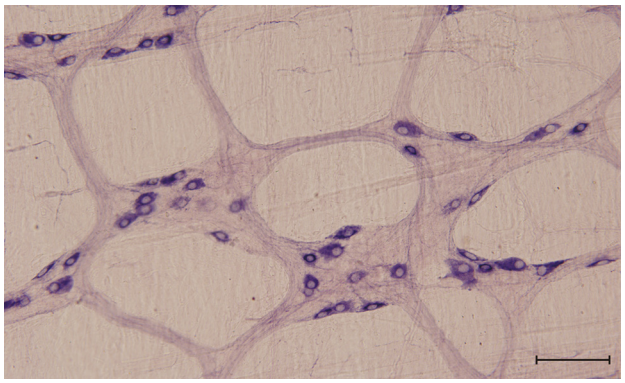

Figure 1 Representative micrograph of whole-mount preparation of the myenteric plexus of the colon from control rat labeled with nicotinamide adenine dinucleotide phosphate-diaphorase. The calibration bar denotes 40 μm.

Quantitative changes of enteric neurons

The combination of intracellular signaling disorders with quantitative and neurochemical changes of the enteric neurons can be related to the neuronal loss and relevant clinical problems of the neurological manifestations of diabetes mellitus, such as dilatation of the stomach, small and large intestines, constipation and diabetic diarrhea[71,72]. A recent study found a significant decrease in ganglion size in diabetic patients compared with normal individuals and enhanced apoptosis of the enteric neurons[72]. There is also evidence of damage to the enteric neurons in animal models of diabetes[73-78].

Subpopulation of nitrergic neurons

Different subpopulations of myenteric neurons are differentially susceptible to the development of neuropathy in diabetes. Zandecki *et al*[18] characterized the myenteric neuropathy in the jejunum of spontaneously diabetic BioBreeding rats. Their results provide evidence for a selective nitrergic motor dysfunction in the jejunum of these diabetic rats. The underlying mechanism involved decreased nNOS protein expression, while the purinergic NANC transmission was not affected.

In animal models of type-1 diabetes, damage of the vagus nerve also contributes to changes in the ENS[79,80]. As nNOS expression is not controlled by the vagus nerve in the jejunum of rat, the nitrergic neuropathy is believed to result from a primary dysfunction in the ENS rather than from vagal dysfunction[18].

Regionality of nitrergic neuropathy

The available reports focusing only on single segments of the GI tract are somewhat contradictory. In our earlier study[22], the streptozotocin- (STZ) induced diabetic rat model was used to investigate the relationship between the deranged gut motility and the segment-specific quantitative changes in the nitrergic myenteric neurons. Additionally, we studied the effectiveness of early insulin replacement to prevent the development of diabetes-induced changes. The NADPH-d-stained cells were considered to be nitrergic neurons when they were double-labeled with HuC/HuD used as a pan-neuronal marker (Figure 2A). The duodenum of the diabetic rats was the only gut segment where the number of nitrergic neurons was decreased, while the total neuronal number was not altered. In the jejunum, ileum and colon, both the total and the nitrergic neuronal cell number decreased significantly (Figure 2B and C). Immediate insulin replacement did not prevent the nitrergic cell loss significantly in the duodenum and jejunum, but it did prevent it significantly in the ileum and colon. These findings comprise the first evidence that the nitrergic neurons located in different intestinal segments exhibit different susceptibilities to a diabetic state and different responsiveness to insulin treatment[22].

Other results also showed that nitrergic neuropathy appears to be more pronounced in the colon compared with the proximal gut[75,81-82]. The strict regionality of pathological processes called attention to the importance of the molecular differences in the neuronal microenvironment along the GI tract. Since myenteric ganglia are not vascularized capillaries, adjacent to them must be responsible to provide the ganglionic microenvironment, including the proper oxidative circumstances.

Sex dependency of enteric neuropathy

Literary data report about sex-dependent sensibility of enteric neurons to the diabetic state[83-84]. Apoptosis of enteric neurons was characteristic in diabetic males, but not in female rats[83]. Another study provides evidence that females may have a greater dependency on the nitrergic mechanisms in health. After induction of diabetes, gastric emptying was delayed in both male and female rats, but females exhibited significantly delayed gastric emptying compared to males. Furthermore, diabetes seems to affect the nitrergic system to a greater extent in females than in males. Together, these changes may account for the greater vulnerability of females to diabetic gastric dysfunction. These data are consistent with clinical observation that diabetic gastroparesis predominantly affects women[84].

Two phases of nitrergic degeneration

Some studies have mentioned an increase in number and size of NOS neurons as well as fiber thickness[85-88]. Cellek's biphasic model of nitrergic neuropathy can offer a good explanation for these contradictory results[21,65]. According to this model, nitrergic neurons innervating the urogenital and GI organs undergo a degenerative process in two phases in diabetes. The first phase is characterized by an insulin-reversible decrease in nNOS expression in the axons, while in the second phase, apoptotic cell death occurs in the nitrergic neurons which is not reversible by insulin treatment.

Effects of oxidative stress

The nitrergic neurons are not a homogeneous cell population. Some of the nNOS-containing neurons also contain heme oxygenase-2 (HO-2). Double-labeling studies

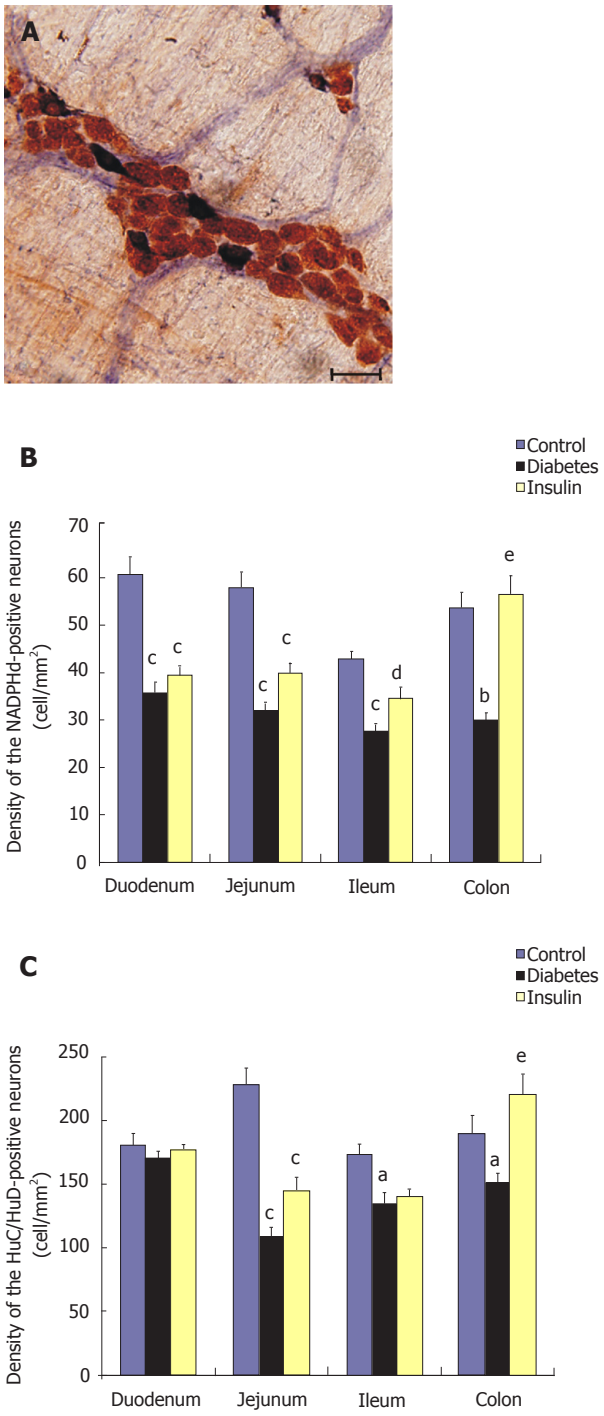

Figure 2 Diabetes-related quantitative changes in the density of total and nitrergic myenteric neurons. Representative micrograph of whole-mount preparation of the myenteric plexus of the duodenum from control rat double-labeled with HuC/HuD and nicotinamide adenine dinucleotide phosphate-diaphorase (A). All nitrergic neurons were double-labeled for the two markers. The calibration bar denotes 50 μm. Densities of HuC/HuD-immunoreactive myenteric neurons (B) and nicotinamide adenine dinucleotide phosphate-diaphorase-positive myenteric neurons (C) in the duodenum, jejunum, ileum and colon of control, diabetic and insulin-treated diabetic rats. Data are expressed as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the control group; $^{d}P < 0.05$, $^{e}P < 0.001$ *vs* untreated diabetic group. NADPH: Nicotinamide adenine dinucleotide phosphate.

revealed that approximately 50% of nNOS-containing neurons also contained HO-2 and that the diabetes-induced change in size was confined to nNOS-immunoreactive neurons that did not contain HO-2. No change in the size and the distribution occurred in neurons in which nNOS and HO-2 were colocalized. This indicates that the antioxidant HO-2 protects those NOS-containing neurons in which it is colocalized against oxidative stress, pointing to the importance of oxidative stress in the development of diabetes-related neuropathies[89].

There is convincing evidence that the generation of reactive oxygen species (ROS) is increased in both type 1 and type 2 diabetes; that the onset of diabetes and its complications are closely associated with oxidative stress; and treatment with antioxidants minimizes or prevents development of these complications in diabetic patients[77-78]. In the non-obese diabetic model of type 1 diabetes, increased oxidative stress has been shown to lead to development of gastroparesis and colonic motor dysfunction[72]. Induction of HO-1, the inducible isoform of HO, at the same time has been identified as an important cellular defence mechanism against oxidative stress[90]. The HO-1 pathway prevents and reverses cellular changes that lead to development of GI complications of diabetes. Induction of HO-1 by hemin decreased ROS, rapidly restored nNOS expression, and completely normalized gastric emptying in mice. Inhibition of HO-1 activity with normal gastric emptying caused development of diabetic gastroparesis[50].

It was earlier found that in the GI tract the colon was more susceptible to damage by oxidative stress[91], and in the colon the apoptosis of the enteric neurons was increased. Detailed analysis of neuronal subtypes from the diabetic and normal colon revealed a selective susceptibility of the inhibitory neuronal sub-populations like nNOS in diabetic patients[90]. The sensitivity of colonic tissue to oxidative stress may arise due to antioxidant or reductant deficiencies. It was observed that colons from diabetic patients had decreased amounts of the non-enzymatic antioxidant reduced glutathione that correlated well with the duration of diabetes. There was also an increased expression of superoxide dismutase, possibly as a compensatory mechanism to match the increase in levels of various free radical scavengers or reductants[72].

ROS can be generated as a result of auto-oxidation of glucose and formation of advanced glycosylation end products (AGEs). AGEs are a group of heterogeneous compounds formed by the non-enzymatic reactions between aldehydic group of reducing sugars with proteins, lipids or nucleic acids. Formation and accumulation of AGEs are related to the aging process and accelerated in diabetes. The pathogenic role of AGEs in vascular diabetic complications is widely recognised[92]. AGEs elicit oxidative stress generation and subsequently cause inflammatory and thrombogenic reactions in various types of cells *via* interaction with a receptor for AGEs. In addition, mitochondrial superoxide generation has been shown to play an important role in the formation and accumulation of AGEs under diabetic conditions[80].

AGEs in the serum and tissues of diabetic rats increases gradually throughout the two phases of diabetes, but the AGEs accumulation in the tissues seems to begin

Table 1 Summary of recent publications on the enteric nervous system in diabetes

Location of change	Type of change	References	Species
Stomach	Gastroparesis, oxidative stress	Choi *et al*[50]	Mouse
Duodenum, jejunum, ileum, colon	Region specific nitrergic neuronal loss, gastrointestinal motility disorders	Izbéki *et al*[22]	Rat
Esophagus, stomach, intestine	Loss of ICCs	Ördög[46]	Human, mouse, rat
Ileum	Loss of enteric neurons	Pereira *et al*[78]	Rat
Jejunum	Decreased NO responsiveness, decreased nNOS protein expression	Zandecki *et al*[18]	Rat
Duodenum	Loss of enteric neurons	De Mello *et al*[88]	Rat
Esophagus, stomach, intestine	Diabetic gastroenteropathy	Ördög *et al*[47]	Human, mouse, rat
Stomach	Gastroparesis, regional injury of ICCs	Wang *et al*[43]	Rat
Colon	Reduction in GFAP and neurotrophins	Liu *et al*[82]	Rat
Small intestine	Loss of enteric neurons, gastrointestinal motility disorders	Nezami and Srinivasan[3]	Human, mouse, rat
Colon	Gastrointestinal motility disorders, loss of enteric neurons, increased oxidative stress	Chandrasekharan *et al*[72]	Human
Stomach	Gastroparesis	Hasler *et al*[106]	Human
Stomach, intestine	Oxidative stress	Kashyap *et al*[90]	Human, mouse, rat
Stomach	Gastroparesis	Tang *et al*[97]	Human
Duodenum, cecum	Loss of enteric neurons	Zanoni *et al*[156]	Rat

ICCs: Interstitial cells of Cajal; NO: Nitric oxide; nNOS: Neuronal nitric oxide synthase; GFAP: Glial fibrillary acidic protein.

at the time point when the nNOS depletion becomes irreversible. This model suggests that irreversible rise in the serum and accumulation of AGEs in the tissues is the trigger for nitrergic apoptosis. The time point where the two phases are separated was called "the point of no return"[65]. The selective nitrergic neuropathy[63] is most probably due to the fact that endogenous NO and accumulated AGEs synergistically cause oxidative stress within the nitrergic neuron, which leads to apoptosis[65].

MOTILITY DISORDERS IN DIABETES

GI motility disorders, such as gastroparesis, constipation and diarrhea, often accompany diabetes, both in patients and in animal models. Population-based studies have shown that 2%-19% of diabetic patients report upper GI symptoms and 48%-65% of those with abdominal symptoms have delayed gastric emptying[93-97].

Motility disorders in diabetes are traditionally considered to originate from visceral autonomic neuropathy, especially changes in vagal innervation. However, increasing evidence from animal models points towards changes in the ENS as the underlying mechanism for these motility disturbances[18,79,98-100]. Changes in adrenergic and cholinergic neurotransmission have been reported[101-102] but recent studies have focused on altered NANC innervation[18].

Nitrergic control was impaired in diabetic rats as a consequence of both decreased smooth muscle responsiveness to NO and decreased nNOS protein expression. Nitrergic enteric neuropathy in diabetes may be a primary dysfunction, occurring independently from vagal dysfunction[18]. Results indicate that the colonic peristaltic reflex is enhanced by impairment of enteric nitrergic inhibitory neurons in spontaneously diabetic rats[75].

Loss of nitrergic neurons in diabetes can result in delayed gastric emptying due to the loss of neurons in the pylorus and accelerated intestinal transit due to the loss of influence of these neurons in the small and large intestine[103].

Diabetic gastroparesis was initially described by Kassander in 1958 as "gastroparesis diabeticorum" in a patient with type 1 diabetes, but it is increasingly being recognized in patients with type 2 diabetes. Gastroparesis is defined as a syndrome characterized by abnormal gastric function resulting in delayed gastric emptying in the absence of mechanical obstruction[44,104]. The pathogenesis of diabetic gastroparesis is multifactorial and results in a neuromyopathy[97]. Most data from recent animal and human studies suggest that the two main findings in diabetic gastroparesis are the loss of ICCs and reduced expression of nNOS[44,50,73,105,106]. Experimental data indicate that in diabetes, increased oxidative stress due to the low HO-1 level in addition to reduced insulin and insulin-like growth factor-1 signaling, not hyperglycemia, is responsible for the loss of the ICCs. The depletion of ICCs causes abnormalities in gastric slow waves, absence of peristalsis and atrophy of gastric smooth muscle[50,97,107].

Our results[22] showed that the STZ-induced diabetic rats displayed faster small intestinal and colonic transit, as observed by others in different rat models of diabetes[108-110]. We therefore infer that our observations in this model with regard to the changes in the total myenteric neurons and the nitrergic subpopulation furnish data on the pathogenesis of diabetic diarrhea, which is a serious complication of diabetes in approximately 10% of diabetic patients.

Colorectal dysfunction is also common in diabetes.

Of patients attending specialized diabetes clinics, up to 60% reported constipation, 22% had diarrhea and 20% had fecal incontinence[111]. In diabetic rodents, constipation was accompanied by reduced neuromuscular neurotransmission in the distal colon, whereas a paradoxical increase in contractile and underlying spike complex activity was noted in the proximal colon. The latter occurred in the absence of reduced inhibitory control and may have reflected functional compensation or a response to small intestinal bacterial overgrowth. ICCs were reduced in the colon of mice with both type 1- and type 2-like diabetes, as well as in type 2 diabetic patients[46,112-115].

CARDIOVASCULAR RISK FACTORS AND IMPAIRED NEURONAL FUNCTIONS

Both type 1 and type 2 diabetes mellitus have long been recognized as an independent risk factor for cardiovascular disease (CVD), including coronary artery disease, stroke, peripheral arterial disease, cardiomyopathy and congestive heart failure. CVD is the leading cause of comorbidity and death in patients with diabetes[116-118]. Vascular complications of diabetes also extend to microvascular disease, manifested as diabetic nephropathy, neuropathy and retinopathy. Chronic hyperglycemia plays a major role in the initiation of diabetic vascular complications[119-120]; however, the mechanisms through which hyperglycemia promotes the development of vascular diseases remain incompletely understood.

Multiple mechanisms for this relationship between glucose and atherosclerosis have been proposed. Hyperglycemia may activate nuclear factor-κB, a key mediator that regulates proinflammatory and proatherosclerotic target genes in endothelial cells, vascular smooth muscle cells and macrophages[121]. Hyperglycemia can also foster the non-enzymatic formation of AGEs, protein cross-linking and ROS formation[122]. Hyperglycemia stimulates oxidative stress, which appears to be a driving force in atherosclerosis[123]. Common final pathways among most, if not all, of these various mechanisms are stimulation of inflammation, arterial remodeling and tissue damage[124,125]. In addition to systemic factors, organ-specific factors also appear to be important in the development of vascular disease. For example, in the kidney, stimulation of mesangial matrix production by hyperglycemia, activation of protein kinase C and an increasing degree of intraglomerular hypertension may contribute to glomerular injury[126]. Other factors associated with the development of vascular disease in type 2 diabetes include impaired endothelial-dependent relaxation, increased proliferation of vascular smooth muscle cells and increased non-enzymatic collagen glycation[127]. Hyperglycemia may also activate matrix-degrading metalloproteinases, enzymes implicated in plaque rupture and arterial remodeling, inducing similar responses in vascular smooth muscles[128].

Although intensive glycemic control has reduced the risks of micro- and macrovascular complications, this strategy is not successful in all patients; therefore, cardiovascular events remain the leading risk factor for mortality of diabetic patients worldwide[129-130]. Glycemic control in the context of type 2 diabetes, as well as pre-diabetes, is also intertwined with cardiovascular risk factors such as obesity, hypertriglyceridemia and blood pressure control[131-133]. Similarly, major issues and concerns have arisen around the cardiovascular safety of antidiabetic therapy[134-136]. Together, these issues have focused attention on the need to understand the cardiovascular effects of current treatments for diabetes and the optimal strategies for care of patients with this disease.

Endothelial dysfunction in the gut wall

Since endothelium is the primary physiological source of endothelial NOS (eNOS) which then produce NO to regulate cardio- and cerebrovascular homeostasis, loss of the modulatory role of the endothelium may be a critical and initiating factor in the development of diabetic vascular disease. Impaired function of the vascular system then leads to ischemia, stroke and consequently hypoxia and neuropathy[137-139]. Because of their dominant clinical incidence, the diabetes-induced alterations in the capillary endothelium of retina[140-143] and renal glomerulus[144,145] have been the focus of a vast number of studies, while except for an early case report on microangiopathy in a bowel biopsy[146], the impact of diabetes on capillaries within the intestinal wall has been almost completely overlooked until now. The myenteric ganglia are not vascularized; accordingly, the capillaries adjacent to the MP have the role to supply them. Therefore, knowing the mechanisms by which diabetes inflicts structural, functional and molecular changes in these capillaries may open new directions in diabetes research and then offer alternative mechanisms to treat the complications associated with hyperglycemia. Due to the growing incidence of insulin resistance, it is becoming increasingly important for clinicians to introduce alternative therapies and be aware of diabetes-related vascular complications[130].

In our ongoing research, we provided evidence[25] that endothelial cells in capillaries adjacent to the MP are direct targets of diabetic damage. The microvessels in a particular gut segment were affected differentially by the pathophysiological conditions, allowing neurons in one intestinal region to survive, while causing them to die in another. Furthermore, we proved that structural and functional alterations which influence the permeability of these capillaries[25] coincide with the enteric neuropathy demonstrated in STZ-induced diabetic rats[22]. Investigations are currently in progress in our laboratory to explain the molecular background of the diabetes-related changes in capillaries supplying the MP.

Vascular permeability and the expression of cell adhesion molecules are regulated by many complex signaling pathways within endothelial cells[138,147,148]. The major negative regulatory protein for eNOS is caveolin-1 (Cav-1). The pathways which involve the regulation of eNOS by Cav-1 in different vascular beds[149] are the focus of

research. Now, we want to know whether the diabetes-induced alterations in the microvasculature of the retina, renal glomerulus and nerves are accompanied by changes in the capillaries supplying the MP and whether such changes can result in an impairment of the strict control of capillary permeability, which then gives rise to the gut region-specific nitrergic neuropathy demonstrated in the MP of rats with STZ-induced diabetes[22].

Although the metabolic and cellular mechanisms leading to severe macro- and microvascular diseases may differ between type 1 and type 2 diabetes, both share a decreased NO bioavailability and altered vascular permeability[150]. A deficit in bioavailable NO could result from an impairment of the eNOS function or the inactivation of NO by oxidative stress. eNOS is a membrane-associated NOS isoform, and the proper localization of eNOS is therefore necessary for its interactions with other regulatory proteins (scaffolds, chaperones and kinases) that fine-tune the cycles of eNOS activation and inactivation[151,152]. Recent studies with Cav-1-deficient mouse models suggest that they may be profoundly important for postnatal cardiovascular functions, including the endothelial barrier function and the regulation of NO synthesis[151-153]. It has also been demonstrated that insulin regulates the distribution of Cav and stimulates the phosphorylation of Cav protein[115].

ENTERIC GLIA

Enteric neurons are surrounded and outnumbered by EGCs. Recent data suggest that EGCs play an important role in the maintenance of tissue integrity in the GI tract[78,82,154]. Several lines of evidence implicate that the secretion of neurotrophic factors by EGCs may be a part of glial regulation of gut homeostasis. The secretion of glia cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and transforming growth factor-beta contribute to the maintenance of endothelial integrity and vasodilatation[82]. Evidence is accumulating that EGCs share the ability of astrocytes to regulate tight-junction integrity and cellular interactions comparable with those maintaining the blood-brain barrier and creating the proper microenvironment for enteric neurons[36,38]. To know the exact mechanisms of how EGCs contribute to gut homeostasis under physiological and pathophysiological conditions is therefore important to work out new therapeutic strategies and to be aware of diabetes-related vascular complications.

Several authors have shown that autonomic neuropathy caused chronically by diabetes mellitus is related to quantitative and morphometric changes in the enteric neurons in various GI segments[22,77,155,156]. However, studies on the number and area of glial cells in diabetes mellitus are scarce. Recent data proves that, unlike the neurons, the diabetic condition in rats did not reduce the glial density per unit area of the intestine. This glial preservation may be attributable to the resistance of the glia cell population and a defense mechanism exerted by glia in an attempt to promote the maintenance of neurons that remain viable after the development of peripheral diabetic neuropathy[78]. Nerve cells cannot synthesize glutathione, the major endogenous cellular antioxidant, because they do not contain the enzyme gamma-glutamyl-cystein-synthetase which is responsible for formation of a peptide bond between cysteine and glutamate[157]. Thus, neurons depend directly on glial cells for glutathione synthesis. This dependence that neurons have on glial cells becomes even more important in diabetes, because changes in glutathione metabolism are common in diabetic patients and associated with reduced levels of these antioxidants[158]. Furthermore, glial cells directly promote neuronal protection by increasing the intracellular content of total glutathione in their own cells. Oxidative stress increases expression of the enzyme gamma-glutamyl-cystein-synthetase in glial cells, which promotes a neuroprotective mechanism by release of glutathione to neurons that survive diabetic neuropathy[159]. This increase in glutathione in glial cells is also a defence mechanism because it protects against the diabetes-induced death of glial cells by inhibition of lipid peroxidation reactions[78].

The area of the glial cells was decreased in the diabetic rats compared to the controls. This decrease may be related to a reduction in the expression of neurotrophic factors or neurotrophins responsible for promoting the survival and maintenance of neurons[78]. This hypothesis is consistent with other studies. The induction of diabetes is associated with a reduction in glial fibrillary acidic protein (GFAP) and neurotrophins expression in the colon, which may affect the role of EGCs and neurotrophins in the enteric plexuses. The changes of GFAP expression in glial cells could be the consequence of unviable extracellular conditions such as hyperosmolarity, low nutrient availability or increased oxidative stress. Immunostaining and western blot showed that diabetes induced a decrease in the intensity of staining of GFAP-positive EGCs and GFAP protein levels at 4 wk and attenuated GFAP expression were more evident at 12 wk[82].

Moreover, mRNA and protein analysis indicated that the levels of NGF were down-regulated in diabetic rats. These findings suggest that the induction of diabetes is associated with a reduction in GFAP and neurotrophins expression in the colon, which may affect the role of EGCs and neurotrophins in the enteric plexuses. This in turn may partly contribute to the physiopathological changes associated with the diabetic state in the GI tract[82]. The neurotrophic factor GDNF reverses hyperglycemia-induced neuronal apoptosis and loss of nitrergic neurons and also improves GI motility in diabetic mice. Therefore, GDNF may be a potential therapeutic target for GI motility disorders in diabetes[103].

NEW STRATEGIES TO IMPROVE GLYCEMIC CONTROL IN DIABETES

New therapies using various drug treatments to improve glycemic control in diabetes have recently been developed or are under development. Current therapies for the

treatment of type 1 diabetes include daily administration of exogenous insulin and less frequently, whole pancreas or islet transplantation. More recently, embryonic or induced pluripotent stem cells have also been examined for their ability to differentiate *in vitro* into pancreatic endocrine cells[160-162]. The first results of glucagonocentric reconstruction of diabetes at the same time open a new perspective over insulin monotherapy of type 1 diabetes. A recent publication[163] proposes that glucagon excess, rather than insulin deficiency, is the main cause of type 1 diabetes. Based on recently accumulated evidence[163,164], it was concluded that glucose-responsive β cells normally regulate juxtaposed α cells and that without intraislet insulin, unregulated α cells hypersecrete glucagon, which directly causes the symptoms of diabetes. Although patients with type 1 diabetes have an absolute deficiency of insulin, the pathogenesis of type 2 diabetes mellitus is associated with relative insulin deficiency and insulin resistance[165-167]. Therefore, in addition to insulin, a number of different classes of medication to treat patients with diabetes have been developed.

There is a growing body of evidence that the incretin hormone, glucagon-like peptide 1 (GLP-1), has profound effects on the GI motor system[168-170]. Moreover, the effects of GLP-1 on GI motility appear to be pivotal to its effect of reducing postprandial hyperglycemia[171-173]. In a recent study, exogenous GLP-1 was able to reduce mouse gastric motility by acting peripherally in the antral region, through neural NO release[174]. It has recently been demonstrated that GLP-1 receptors are expressed in the enteric neurons. Furthermore, 27% of GLP-1 receptor immunoreactive neurons in the duodenum and 79% of these neurons in the colon are co-expressed with nNOS[175].

Due to its promising potential in the treatment of type 2 diabetes and related intestinal motility disorders, the incretin-based therapies have been the focus of much interest during the last years[176-179]. Incretins, which are released by enteroendocrine cells in the intestine in response to a meal, have been implicated in contributing to the pathogenesis of type 2 diabetes mellitus. Injectable GLP-1 receptor agonists and orally administered dipeptidyl peptidase-4 (DPP-4) inhibitors have been developed[180,181] and introduced into clinical practice to specifically address the blunted incretin responses in patients with diabetes type 2. The GLP-1 receptor agonists potentiate insulin secretion, inhibit glucagon release, delay gastric emptying and reduce appetite. The DPP-4 inhibitors primarily improve insulin secretion and inhibit glucagon release.

In diabetic rats, a DPP-4 inhibitor improved the thickening of the glomerular basement membrane[182] which is the histological hallmark of diabetic microangiopathy. GLP-1 administration also decreases the damage of alveolar capillary basal lamina in rats with spontaneous type 2 diabetes mellitus[183]. The use of these drugs is also associated with improvements in blood pressure, diabetic dyslipidemia and myocardial function[184-186]. Therefore, they have a potential role to reduce the cardiovascular risk factors, a major cause of mortality in patients with diabetes.

CONCLUSIONS AND PERSPECTIVES

Intestinal region-specific selective loss of enteric neurons in rat models of diabetes mellitus indicates the importance of the neuronal microenvironment in the pathogenesis of diabetic enteric neuropathy. Therefore, among the most important players of enteric microenvironments, capillary endothelium and EGCs have received much attention in recent years. Studies in humans and in animal models indicate that the mechanisms of endothelial dysfunction differ according to the diabetic model and the vascular bed under study. Therefore, different animal models and different vascular beds must be considered in future studies in order to be able to draw general conclusions on the anatomical, physiological and molecular mechanisms leading to the development of diabetic enteric neuropathies that generally appear as a consequence of vascular complications.

The gut region-specific neuronal and vascular damage demonstrated in STZ-induced diabetic rats[22] leads to the question of why the enteric neurons, glial cells and microvessels in the different intestinal segments are affected differentially by the diabetic condition. Since correlations have been suggested between the host's health and the GI tract microbiota, numerous investigations in recent years have focused on the connection between the GI tract microbiota and metabolic diseases. Most recent findings[187-189] provide a sufficient basis for the speculation that the different degrees of susceptibility of enteric neurons and microvessels to a pathological stimulus such as hyperglycemia might be related to the prevalence of bacteria in the different parts of the GI tract. Accordingly, the differences in prevalence of bacteria in different gut segments[188,190] are influenced by the oxygen supply of the small and large intestine[191]. Knowledge of species and functional composition of the gut microbiome is rapidly increasing thanks to technological advances in culture independent methods[189,192-195]. The human GI tract is dominated by anerobic bacteria mainly in the distal part of the gut[190]. We presume that, due to the adequate oxidative environment in the proximal intestine, the enteric neurons or capillaries there can tolerate hyperglycemia-related oxidative stress better and for a longer time than they can in the colon, where the basal oxygen supply is far from optimal.

The intestinal microbiota have been shown to be different in composition and causally linked to metabolic diseases such as diabetes and obesity in humans and mice[187,196-199]. Furthermore, the divergences from the core microflora may define the status of disease[200-201]. The development of diabetes type 1 in rats was reported to be associated with higher amounts of *Bacteroides ssp.*[202]. It has been proposed that the gut microbiota directed increased monosaccharide uptake from the gut and

instructed the host to increase hepatic production of triglycerides associated with the development of insulin resistance[203]. Larsen *et al*[187] demonstrated that type 2 diabetes is also associated with compositional changes in the intestinal bacteria. Accordingly, their results show that the relative abundance of Firmicutes was significantly lower, while the proportion of Bacteroidetes and Proteobacteria was somewhat higher in diabetic persons compared to non-diabetics.

The lactate- and butyrate-producing bacteria in a healthy gut induce a sufficient amount of mucin synthesis to maintain gut integrity. In contrast, non-butyrate-producing lactate-utilizing bacteria prevent optimal mucin synthesis, as identified in autoimmune subjects[204]. Obese and diabetic mice display enhanced intestinal permeability by reducing the expression of genes coding for two tight junction proteins, ZO-1 and occludin[198]. It was proved that prebiotic modulation of gut microbiota lowers intestinal permeability by increases in endogenous GLP-2 production, thereby improving gut barrier function, glucose-tolerance and low-grade inflammation[198-199].

In order to precisely determine the role of the gut microbiota in the development of metabolic diseases, among others, diabetes mellitus type 1 and type 2, and provide new therapeutic strategies, it is crucial to collect more detailed information on the host-microbial homeostasis.

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ORIGINAL ARTICLE

Curcumin attenuates Nrf2 signaling defect, oxidative stress in muscle and glucose intolerance in high fat diet-fed mice

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Author contributions: He HJ performed the experiments throughout the study and wrote the draft of this manuscript; Wang GY and Gao Y participated in the intraperitoneal glucose tolerance test experiments and mice tissue collection; Ling WH provided his advice on HFD feeding and the dosage of curcumin in this study; Yu ZW designed the study, provided technical guidance and edited this paper; Jin TR coordinated the study, provided important guidance and final editing of the manuscript.

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Abstract

AIM: To investigate the signaling mechanism of antioxidative action by curcumin and its impact on glucose disposal.

METHODS: Male C57BL/6J mice were fed with either a normal diet (n = 10) or a high fat diet (HFD) (n = 20) to induce obesity and insulin resistance. After 16 wk, 10 HFD-fed mice were further treated with daily curcumin oral gavage at the dose of 50 mg/kg body weight (BW) (HFD + curcumin group). After 15 d of the curcumin supplementation, an intraperitoneal glucose tolerance test was performed. Fasting blood samples were also collected for insulin and glucose measurements. Insulin-sensitive tissues, including muscle, adipose tissue and the liver, were isolated for the assessments of malondialdehyde (MDA), reactive oxygen species (ROS) and nuclear factor erythroid-2-related factor-2 (Nrf2) signaling.

RESULTS: We show here that in a HFD mouse model, short-term curcumin gavage attenuated glucose intolerance without affecting HFD-induced BW gain. Curcumin also attenuated HFD-induced elevations of MDA and ROS in the skeletal muscle, particularly in its mitochondrial fraction, but it had no such an effect in either adipose tissue or the liver of HFD-fed mice. Correspondingly, in skeletal muscle, the levels of total or nuclear content of Nrf2, as well as its downstream target, heme oxygenase-1, were reduced by HFD-feeding. Curcumin intervention dramatically reversed these defects in Nrf2 signaling. Further analysis of the relationship of oxidative stress with glucose level by a regression analysis showed a positive and significant correlation between the area under the curve of a glucose tolerance test with MDA levels either in muscle or muscular mitochondria.

CONCLUSION: These findings suggest that the short-term treatment of curcumin in HFD-fed mice effectively ameliorates muscular oxidative stress by activating Nrf2 function that is a novel mechanism for its effect in improving glucose intolerance.

Key words: Oxidative stress; Insulin resistance; Glucose tolerance; Nuclear factor erythroid-2-related factor-2; Curcumin; Mitochondria

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INTRODUCTION

Obesity is associated with a high incidence of many metabolic disorders, including type 2 diabetes mellitus and cardiovascular diseases[1,2]. Insulin resistance is recognized as a core mechanism for these obesity-related diseases. Extensive recent studies have shown that chronic activation of inflammatory signaling, endoplasmic reticulum (ER) stress and mitochondrial dysfunction are among the etiological factors of insulin resistance[3,4]. In addition, the causal relationship between oxidative stress and the development of insulin resistance[5-7] has been suggested for a long time, while it has gained much more attention recently due to the recognition of the involvement of mitochondrial-derived oxidative stress in the etiology of insulin resistance[4,8]. Over nutrition supply or high fat diet (HFD) consumption lead to increased mitochondrial oxidative phosphorylation and inevitable reactive oxygen species (ROS) over-production[7,8]. Mammals however, have evolved endogenous anti-oxidative systems to prevent oxidative stress and maintain insulin sensitivity.

The transcription nuclear factor erythroid-2-related factor-2 (Nrf2) plays a major role in maintaining redox balance. In a resting cell, Nrf2 molecules mainly reside in cell cytosol and are anchored with its negative regulator Kelch-like ECH-associated protein 1 (Keap1). The coupling between Nrf2 and Keap1 leads to Nrf2 proteasomal degradation[9]. When oxidative stress occurs, Nrf2 and Keap1 will be separated, leading to the elevation of free Nrf2 level and the increase of Nrf2 nuclear translocation. Nuclear Nrf2 will then bind to the consensus nucleotide sequence, namely antioxidant response element (ARE), in the promoter regions of a battery of genes that encode antioxidant enzymes[9,10]. Since oxidative stress is an important etiological factor in aging, inflammation and tumor growth, it is not surprising that Nrf2 can modulate the progress of these diseases[11,12].

Interestingly, certain natural phyto-compounds, including curcumin, are Nrf2 activators and have a potential therapeutic effect for diseases, including inflammation, tumor, heart failure, neurodegenerative diseases and ischemia[13-16]. Curcumin has also been shown to sensitize insulin action in HFD-fed diabetic models[17]. The understanding of molecular mechanisms underlying the insulin sensitizing action by curcumin is essential for the generation of interventional approaches. Most *in vivo* investigations have been conducted to explore long-term effect of curcumin and its beneficial effect on insulin signaling mainly *via* reducing body weight (BW) gain and inhibiting inflammatory reactions[17,18]. However, in spite of the documented anti-oxidative function of curcumin[14], no attempt has been made to correlate the role of curcumin-mediated anti-oxidative function and its impact on insulin sensitivity. Since curcumin has been shown to activate Nrf2 in cultured cells[15], here we asked whether curcumin activates Nrf2 system *in vivo* and whether the activation leads to improved insulin signaling. To investigate whether the beneficial effect of curcumin can be achieved independent of its BW lowering effect, we delivered a low dose of curcumin by gavage short-term to avoid BW change.

MATERIALS AND METHODS

Materials

Male C57BL/6J mice (8 wk of age) were purchased from the Laboratory Animal Center of Sun Yat-sen University. Curcumin (curcuminoid content ⩾ 94%), common chemicals and protease inhibitors were obtained from Sigma Chemical Company (St Louis, MO). Normal diet (ND) (8% calories from fat) and HFD (60% calories from fat) were provided by Guangdong Animal Center, (Guangzhou, Guangdong, China). Nrf2 antibody was from Santa Cruze Biotechnology (Santa Cruz, CA). Heme oxygenase-1 (HO-1) and β-actin antibodies were from the Proteintech Group (Chicago, IL). Histone H_3 antibody was obtained from Cell Signaling Technology (Denvers, MA, United States). Bicinchoninic acid (BCA) assay kit was purchased from Pierce Bio-technology, Inc. (Rockford, IL). Enhanced chemiluminescence (ECL) was purchased from Thermo Scientific (Rockford, IL). Blood glucose meter was obtained from ACON Laboratories, Inc. (San Diego, CA). ROS assay kit was purchased from GENMED Scientifics (Shanghai, China). Malondialdehyde (MDA) assay kit was purchased from ZeptoMetrix (Buffalo, NY).

Animal care and treatment

The animal experiments were performed in accordance with the Guide for Care and Use of Experimental Animals (Sun Yat-sen University, SYSU). Thirty mice were housed in an environmentally controlled room at 22 ± 2.0 ℃ and 50% ± 5% humidity with a 12-h: 12-h light/dark cycle. The mice had access to food and water *ad libitum*. After a one week adaptive period, the mice were randomly divided into two weight-matched groups. Ten mice were fed with ND and the rest of them (20 mice) were fed with HFD. BW was measured weekly. After 16 wk of feeding, HFD-fed mice were further divided into two weight-matched groups, e.g., HFD group (n = 10) and HFD plus curcumin treated group (n = 10). Curcumin was given daily by oral gavage at the dose of 50 mg/kg BW in 1% carboxymethyl cellulose buffer solution for 15 d. Mice in the ND and HFD group were gavaged with vehicle only. For blood sample and tissue collection, all mice were euthanized after fasting for 6 h. Blood samples were taken and centrifuged at 4 ℃ at 3000 rpm for 10 min for collecting the serum. Meanwhile, skeletal muscle in quadriceps, livers and epididymal fat pads were rapidly isolated, followed by immediate freezing in liquid nitrogen and then stored at -80 ℃before further analyses.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test was performed as previously described[19]. Briefly, mice were fasted overnight, followed by glucose (1 g/kg) injection intraperitoneally. Blood samples collected from a tail vein were used for glucose measurement.

Mitochondrial and nuclear fractionation

Nuclear and mitochondrial fractionation were performed as previously described[20,21] with a slight modification. About 30 mg skeletal muscle was homogenized in 1 mL ice-cold buffer containing 20 mmol/L HEPES (pH 7.4), 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol and the protease inhibitors [2 μg/mL aprotinin, 5 μg/mL leupeptin and 2 mmol/L phenylmethyl sulphonyl fluoride (PMSF)]. Following the homogenization procedure, samples were incubated on ice for 20 min. Centrifugation was then carried out at 720 × *g* at 4 ℃ for 5 min. The nuclear pellet was dispersed by the buffer and passed through a 21G needle 20 times. The samples were then centrifuged again at 720 × *g* at 4 ℃ for 10 min. After removing the supernatant, the nuclear pellet was re-suspended in 50 μL nuclear lysis buffer containing 20 mmol/L Tris (pH 7.5), 137 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1% Nonidet P-40 (NP-40), 2 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS) and the aforementioned protease inhibitors. The nuclear pellet was sonicated briefly for 3 s and saved for western blotting. For mitochondrial fractionation whole cell lysate after removing the nuclei was centrifuged at 10 000 × *g*, 4 ℃ for 10 min and saved the pellet as mitochondrial fraction at -80 ℃.

MDA analysis in serum and tissues

MDA level was determined by the thiobarbituric acid (TBA) method[22]. TBA reaction was performed according to manufactory guidance. To assess MDA level in serum, 100 μL serum was used. For tissue MDA assay about 30 mg of tissues (skeletal muscle, liver or adipose tissue) or mitochondria extracted from 30 mg skeletal muscle were homogenized in 300 μL ice-cold PBS. TBA reaction was performed according to manufactory guidance. Briefly, samples were incubated with TBA and SDS at 95 ℃ for 1 h, followed by a centrifugation at 800 × *g* for 10 min. Supernatants were transferred to a 96-well plate and the absorbance was measured at 532 nm. The protein concentration in samples was determined by a BCA assay kit. Serum MDA level was calculated according to the formula provided by the company guidance, i.e., serum MDA (nmol/mL) = (sample OD value-background OD)/(Standard OD-blank OD) × standard concentration (10 nmol/mL) × sample dilution times, while tissue MDA level after the calculation was further corrected by sample protein concentration (mg protein/mL). MDA equivalents were expressed as nmol/mg tissue protein or nmol/mL serum.

ROS measurements in tissues

Skeletal muscles were homogenized and ROS level analysis was performed using 2',7'-dichlorofluorescein diacetate fluorescent dye as a probe and fluorescence density was measured at 490/520 nm according to manufactory guidance and the measured values of optical density [a relative fluorescence unit (RFU)] were corrected by the protein concentrations of samples and were expressed as RFU/μg protein.

Insulin and glucose measurements and homeostasis model assessment - insulin resistance index

After the mice were fasted for 6 h, blood samples were collected. To assess plasma insulin levels, an ELISA based method (Mercodia AB, Uppsala, Sweden) was used, the method was according to manufactory guidance and expressed as μg/L. Plasma glucose assay levels were determined using a glucose assay kit from Sigma-Aldrich (Saint Louis, United States), with the method provided by the manufacturer. The level of the original plasma glucose concentration was measured at 540 nm and expressed as mg/dL. Homeostasis model assessment- insulin resistance index (HOMA-IR) was calculated as [fasting plasma glucose (mmol/L) × fasting serum insulin (μIU/mL)] /22.5[23].

Tissue protein preparation and western blotting

Methods for tissue protein preparation and western blotting were performed as described[19]. A fraction of liver, adipose tissue and skeletal muscle were homogenized in ice-cold lysis buffer containing 20 mmol/L Tris (pH 7.5), 137 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1% NP-40, 2 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 10% glycerol, 2 μg/mL aprotinin, 5 μg/mL leupeptin and 2 mmol/L PMSF. The homogenates were then sonicated on ice cold conditions three times for 20 s and centrifuged at 12 000 rpm for 10 min at 4 ℃. The supernatant was collected. The protein concentration was determined by BCA assay kit. Equal amounts of proteins were used for western blot analysis. The proteins were heated at 95 ℃ for 5 min in SDS sample loading buffer and underwent SDS-PAGE analysis as described in the following. The cellular proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Following the transfer, the membranes were incubated for 1.5 h at room temperature in the buffer containing 25 mmol/L Tris (pH 7.6), 154 mmol/L NaCl, 0.1% Tween-20 and 5% skimmed milk. The membranes were then probed with antibody overnight at 4 ℃. The membranes were washed and incubated with secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were visualized with ECL. The intensities of the bands were quantified by phosphorimager analysis using NIH image software.

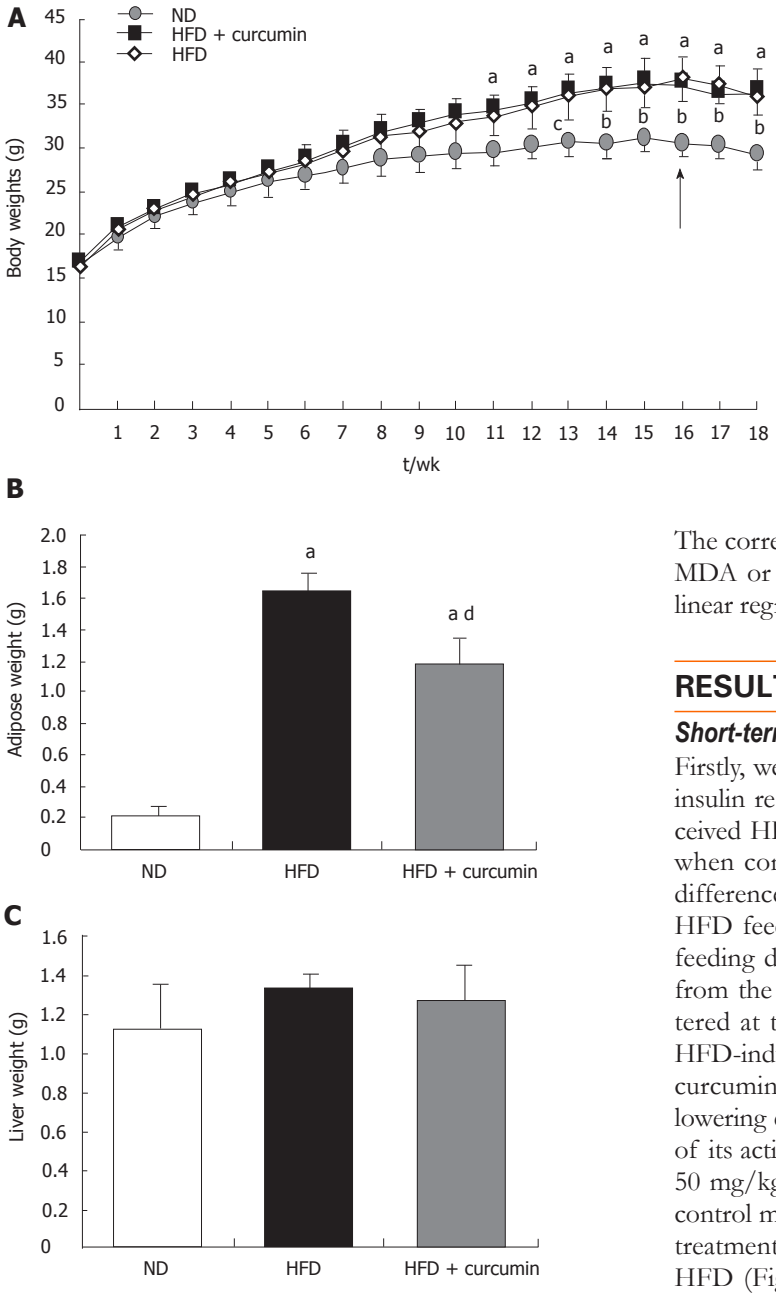

Figure 1 Effects of short term curcumin gavage on weights of whole body, epididymal fat tissue and liver. A: Body weight. The body weight of mice was measured weekly following a normal diet or high fat diet feeding during the period of 18 wk. The arrow indicates the starting time of curcumin gavage; B: Weight of epididymal adipose tissue; C: Liver weight. The mice were fed with either a normal diet (ND, n = 10) or high fat diet (HFD, n = 20) for 16 wk. The HFD fed mice then received gavage of either curcumin (50 mg/kg per day, n = 10) or vehicle (1% carboxymethyl cellulose buffer, n = 10) for 15 d. The ND fed mice were also gavaged with the vehicle. The mice were sacrificed for tissue isolation and weighing. $^{a}P < 0.001$, HFD or HFD + curcumin *vs* ND; $^{b}P < 0.001$, HFD + curcumin *vs* ND; $^{c}P < 0.01$ HFD + curcumin *vs* ND; $^{d}P < 0.05$, HFD + curcumin *vs* HFD. Four mice from HFD and four mice from HFD + curcumin group died during the gavage procedure.

Statistical analysis

All the values were expressed as mean ± SE. Statistical comparisons of means among three groups were carried out by using analysis of variance and the P value less than 0.05 was considered to be statistically significant. The correlation between area under the curve (AUC) and MDA or ROS was tested with the method of multiple linear regressions.

RESULTS

Short-term curcumin treatment does not alter BW

Firstly, we fed the mice with HFD to induce obesity and insulin resistance. As shown in Figure 1A, mice who received HFD feeding exhibited a more rigorous BW gain when compared with the mice of the ND group. The difference reached statistical significance at 11 wk after HFD feeding. Curcumin was supplemented after 16 wk feeding during HFD consumption, which was different from the previous studies when curcumin was administered at the beginning of HFD feeding and prevented HFD-induced obesity[17,18]. A short-term and low dose of curcumin administration was designed to avoid its BW lowering effect in order to detect the primary mechanism of its action. We gavaged a group of HFD-fed mice with 50 mg/kg BW of curcumin consecutively for 15 d. The control mice received the vehicle solution. This curcumin treatment did not alter BW gain in mice while fed with HFD (Figure 1A). To further detect its effect on obesity, the weight of epididymal fat pads (an indicator of visceral fat mass) was measured. We observed that HFD induced about an 8-fold increase in the weight of epididymal fat pads compared to ND mice (ND: 0.21 ± 0.07 g *vs* HFD: 1.65 ± 0.12 g, $P < 0.001$, Figure 1B) , while curcumin gavage only moderately reduced the weight of epididymal fat in mice who received HFD (HFD + curcumin: 1.19 ± 0.2 g *vs* HFD: 1.65 ± 0.12 g, $P < 0.05$, Figure 1B) and compared with mice in the ND group, the increase of the weight of epididymal fat in curcumin-treated mice was still about 5-fold (ND: 0.21 ± 0.07 g *vs* HFD + curcumin: 1.19 ± 0.2 g, $P < 0.001$, Figure 1B). We also measured the weight of the liver and there was no statistical difference among the three groups (Figure 1C).

Curcumin improves glucose disposal and insulin sensitivity

As indicated previously, curcumin administration signifi-

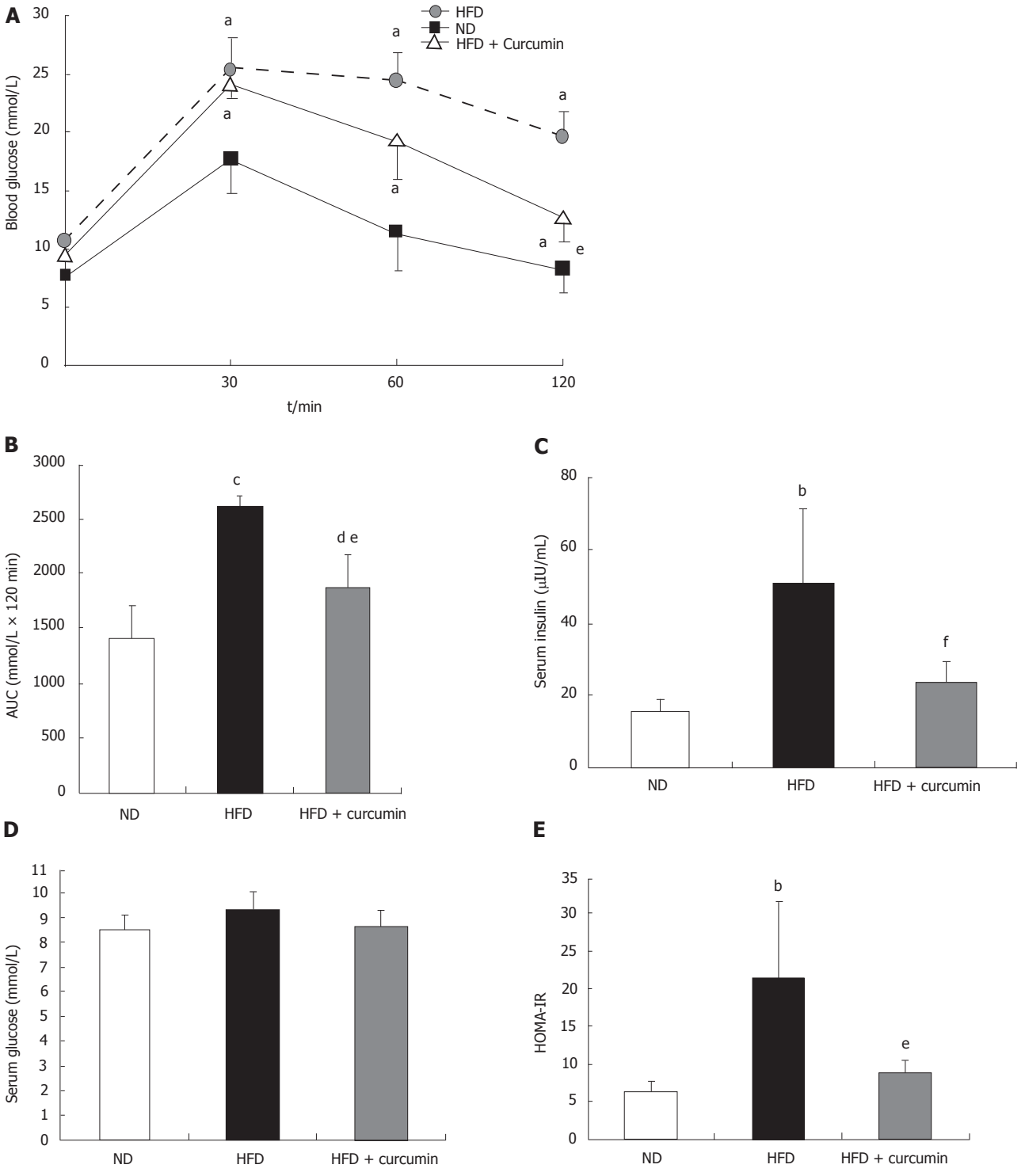

Figure 2 Effects of short term curcumin gavage on glucose tolerance and insulin sensitivity. A: Intraperitoneal glucose tolerance test in the normal diet group (n = 10), high fat diet group (n = 6) and high fat diet plus curcumin group (n = 6); B: Area under the curve of glucose tolerance test; C: Area under the curve; D: Fasting plasma glucose levels; E: Homeostasis model assessment - insulin resistance index values. The mice were fed with different diets and gavaged with curcumin as indicated in Figure 1. At the end of 18 wk following normal diet (ND), high fat diet (HFD) and HFD+curcumin treatments, intraperitoneal glucose tolerance test was performed and blood glucose levels were measured. [a]P < 0.001, HFD or HFD + curcumin *vs* ND at each time point; [b]P < 0.01, HFD *vs* ND; [c]P < 0.001, HFD *vs* ND; [d]P < 0.05, HFD + curcumin *vs* ND; [e]P < 0.01, HFD + curcumin *vs* HFD; [f]P < 0.05, HFD+curcumin *vs* HFD. AUC: Area under the curve; HOMA-IR: Homeostasis model assessment- insulin resistance index.

cantly prevented HFD-induced insulin resistance by long-term supplementation[17]. To assess whether curcumin could reverse insulin insensitivity in an established obese model or to test its treatment efficacy, we performed an intraperitoneal glucose tolerance test (IPGTT). As shown in Figure 2A, after 18 wk feeding of HFD, glucose levels following the i.p. injection were significantly higher when compared with those in ND-fed mice. Curcumin treat-

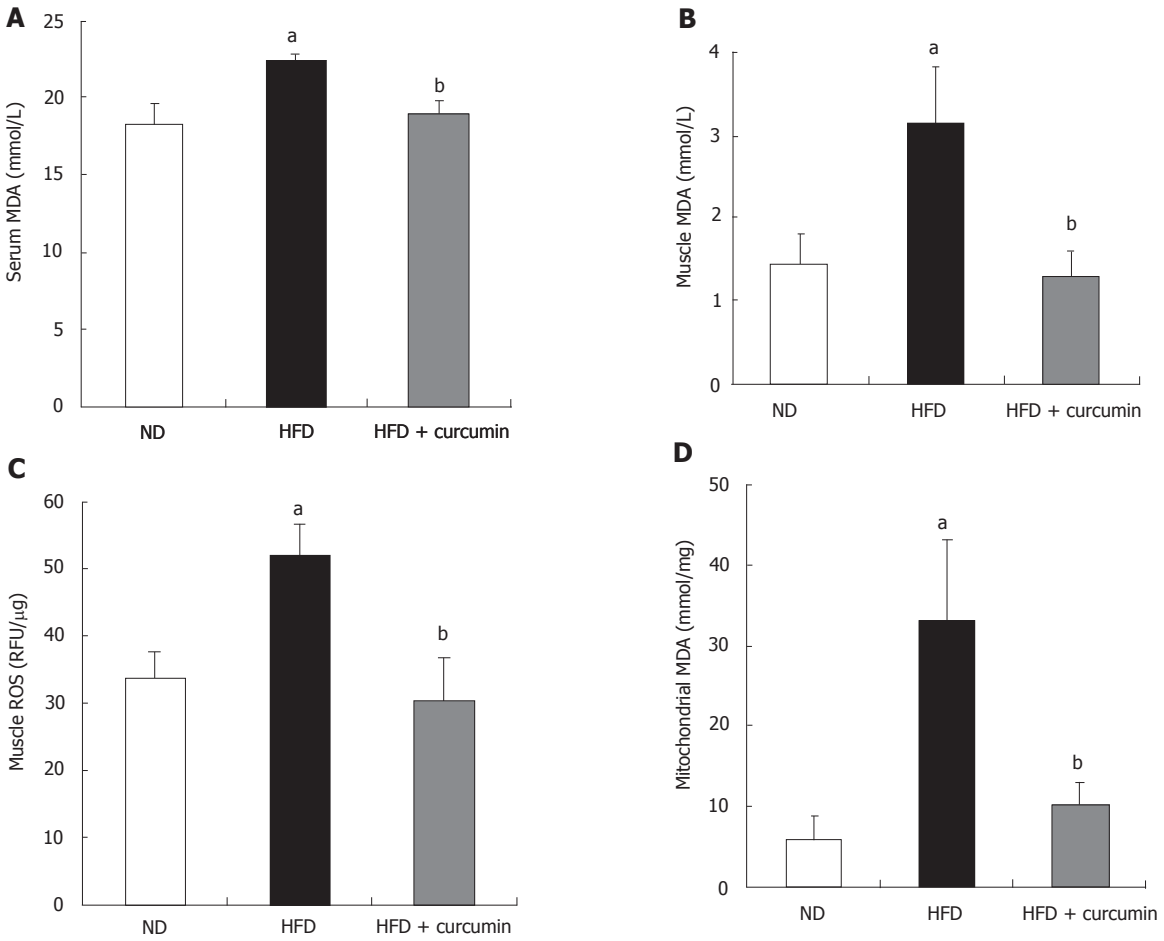

Figure 3 Effects of short term gavage of curcumin on malondialdehyde levels. A: Serum malondialdehyde levels; B: Malondialdehyde levels in skeletal muscle; C: Reactive oxygen species level in whole cell lysates of skeletal muscle; D: Malondialdehyde levels in mitochondria of skeletal muscle. The mice were fed with indicated diets and gavaged with curcumin or vehicle. At the end of 18 wk following normal diet (ND, $n = 10$), high fat diet (HFD, $n = 6$) and HFD + curcumin ($n = 6$) treatments, the mice were sacrificed for blood and tissue collections. Serum was isolated, tissues were homogenized and mitochondria were isolated. Malondialdehyde assessments or reactive oxygen species assay were performed as described in "Materials and Methods". [a]$P < 0.001$, HFD *vs* ND; [b]$P < 0.001$ HFD + curcumin *vs* HFD; [c]$P < 0.01$, HFD + curcumin *vs* HFD. MDA: Malondialdehyde; ROS: Reactive oxygen species.

ment reduced blood glucose levels (Figure 2A). The analysis of AUC of the IPGTT during the tested 120 min also showed that HFD induced about a 2-fold increase in the AUC index compared to that in ND mice, while curcumin normalized this elevation by more than 50% (Figure 2B). Furthermore, in order to assess whether insulin sensitivity was enhanced by curcumin, we measured fasting glucose and insulin levels (Figure 2C and D) and calculated the HOMA-IR index (Figure 2E). We found that HFD induced almost a 3-fold increase in this index while curcumin significantly reversed this abnormality. These results indicate that the effect of curcumin on improving glucose disposal is *via* enhancing insulin action and this action of curcumin in HFD-fed mice is disposable with its effect on attenuating BW gain.

Curcumin ameliorates HFD-induced oxidative stress in skeletal muscle and mitochondria

Increased evidence indicates that oxidative stress plays a causal role in the development of insulin resistance[7,24,25]. We tested whether HFD and curcumin alter redox balance by assessing MDA levels, a stable indicator of oxidative stress[22]. As shown in Figure 3A, HFD induced approximately 20% elevation of serum MDA level, while short-term curcumin gavage completely reversed this elevation (HFD: 22.24 ± 0.50 nmol/mL *vs* HFD + curcumin: 18.82 ± 0.91 nmol/mL, $P < 0.01$). We then assessed MDA levels in the insulin sensitive tissues. MDA level in the skeletal muscle of HFD-fed mice was increased approximately 2-fold when compared with that in the ND mice (Figure 3B). Curcumin administration decreased muscular MDA content to the level that was comparable with that in the ND mice (Figure 3B). Furthermore, muscular ROS content was increased about 40% by HFD feeding while curcumin treatment completely blocked this elevation (ND: 33.61 ± 4.03 RFU/μg *vs* HFD: 51.7 ± 4.92 RFU/μg *vs* $P < 0.001$; HFD: 51.7 ± 4.92 RFU/μg *vs* HFD + curcumin: 30.18 ± 6.66 RFU/μg, $P < 0.001$, Figure 3C).

Since mitochondrial oxidative stress plays a major causative role in insulin resistance in the condition of nutrition over consumption[4,8], we measured the MDA level in the mitochondrial fraction of skeletal muscles.

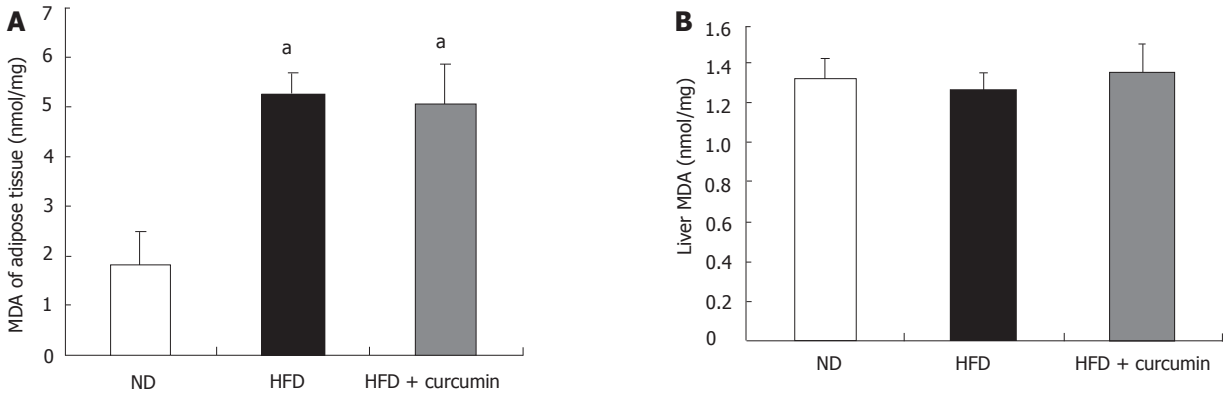

Figure 4 Effects of curcumin on adipose and liver malondialdehyde levels. A: Malondialdehyde levels in adipose tissue; B: Malondialdehyde levels in liver. The mice were fed with indicated diets and gavaged with curcumin or vehicle. At the end of 18 wk following normal diet (ND, n = 10), high fat diet (HFD, n = 6) and HFD + curcumin (n = 6) treatments, the mice were sacrificed, tissues were homogenized and malondialdehyde levels were measured as indicated in the "Materials and Methods". $^{a}P < 0.001$, HFD or HFD+curcumin *vs* ND. MDA: Malondialdehyde.

Indeed, the MDA level in the mitochondrial fraction was pronouncedly elevated in the HFD-fed mice (4-fold elevation compared to ND mice) and curcumin significantly attenuated the effect of HFD (HFD: 33.07 ± 10.16 nmol/mg *vs* HFD + curcumin: 10.05 ± 2.90 nmol/mg, $P < 0.001$, Figure 3D). We, however, did not detect a change of MDA level in liver or adipose tissue by curcumin gavage (Figure 4). By performing a regression analysis on the muscular MDA level and the AUC value in IPGTT, we found that there was a significantly positive correlation between them ($P < 0.05$). The regression analysis was also performed on the muscular mitochondrial MDA level and the AUC value in IPGTT. A more significantly positive correlation was observed ($P < 0.001$). These data suggest the existence of a tight link between muscular mitochondrial redox balance and the ability of the whole body on glucose disposal.

Curcumin activates Nrf2 signaling in skeletal muscles

Curcumin has been shown to activate the Nrf2 system in cell culturing systems[15]. To further explore the potential mechanism of an anti-oxidative effect of curcumin, we determined whether curcumin stimulates Nrf2 signaling *in vivo*. We prepared whole lysates of tissues from the three groups of mice and examined the levels of Nrf2 as well as one of its target gene products, HO-1 by western blotting. As shown in Figure 5A, when compared with the control ND mice, HFD feeding reduced Nrf2 level approximately 50% in muscles, whereas curcumin administration significantly attenuated the repressive effect of HFD (Figure 5A). We however, did not see an apparent change in the protein levels of Nrf2 and HO-1 in the liver and the fat tissue by this short-term curcumin treatment (Figure 5C and D).

Consistently, Nrf2 content in the nuclei of skeletal muscle was reduced in HFD mice while curcumin treatment reversed this reduction (Figure 5B). Furthermore, HFD feeding led to a reduced level of HO-1 whereas curcumin significantly reversed this reduction. These results collectively suggest that HFD feeding impairs the function of Nrf2 system while short-term treatment with curcumin significantly activates the Nrf2-ARE signaling pathway in skeletal muscles of HFD-fed mice.

DISCUSSION

Long-term curcumin administration in mice has been shown to improve insulin signaling and glucose disposal by attenuating inflammation and obesity[17,26]. However, it is not clear whether curcumin could exert its insulin-sensitizing action by anti-oxidative stress. Particularly, very few studies have been conducted to examine the acute effect of curcumin in an already insulin resistant model. We showed here that short-term curcumin gavage improved glucose disposal in a HFD-induced mouse model in the absence of an apparent change of BW. We then observed that short-term curcumin administration ameliorated oxidative stress in both serum and muscles of HFD mice, particularly in muscular mitochondria. Furthermore, we detected that these beneficial effects were accompanied by a reversal of impaired Nrf2 signaling, including increased Nrf2 expression, its nuclear location and the expression of the target antioxidant enzyme, HO-1. We hence suggest that the activation of the major anti-oxidative defense machinery Nrf2 in muscle is a novel mechanism for curcumin in the treatment of insulin resistance and associated metabolic disorders.

In a HFD-induced insulin resistant model, several mechanisms have been proposed to explain the causal role of HFD consumption in the development of insulin insensitivity. Inflammation, ER stress and mitochondrial dysfunction are all reported to be involved[3,6-8]. However, these abnormalities could be the chronic etiology factors of insulin resistance. It is still not known which factor initially induces insulin resistance. It is reasonable to believe that in the condition of nutrition over supply, mitochondrial oxidative phosphorylation metabolism would produce a larger amount ROS and if anti-oxidative machinery could not eliminate the mitochondrial derived oxidants, oxidative stress will occur, resulting in insulin

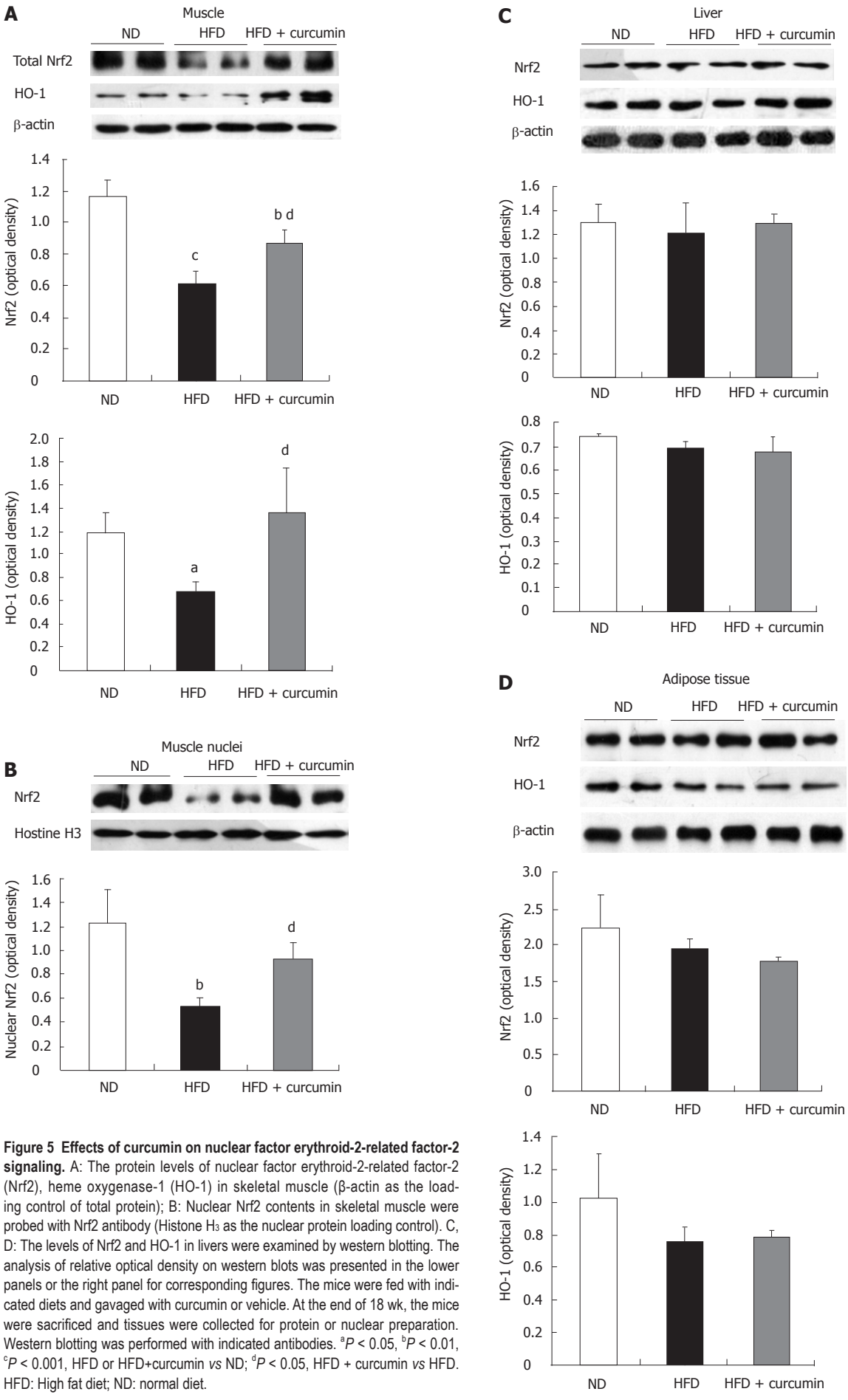

Figure 5 Effects of curcumin on nuclear factor erythroid-2-related factor-2 signaling. A: The protein levels of nuclear factor erythroid-2-related factor-2 (Nrf2), heme oxygenase-1 (HO-1) in skeletal muscle (β-actin as the loading control of total protein); B: Nuclear Nrf2 contents in skeletal muscle were probed with Nrf2 antibody (Histone H_3 as the nuclear protein loading control). C, D: The levels of Nrf2 and HO-1 in livers were examined by western blotting. The analysis of relative optical density on western blots was presented in the lower panels or the right panel for corresponding figures. The mice were fed with indicated diets and gavaged with curcumin or vehicle. At the end of 18 wk, the mice were sacrificed and tissues were collected for protein or nuclear preparation. Western blotting was performed with indicated antibodies. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, HFD or HFD+curcumin *vs* ND; $^{d}P < 0.05$, HFD + curcumin *vs* HFD. HFD: High fat diet; ND: normal diet.

resistance. In support, recent investigations have indicated that muscle mitochondrial derived ROS can acutely impair glucose disposal[27,28] and the administration of mitochondrial specific antioxidant corrects this defect[7,25]. Interestingly, curcumin has an anti-oxidant effect and *in vitro* studies have indicated its action on triggering Nrf2 signaling[14,15]. We therefore proposed the working hypothesis of this study that short-term curcumin supplementation could improve glucose homeostasis in HFD mice by its anti-oxidative action *via* Nrf2 activation. Firstly, we examined the redox status in the three groups of mice by MDA assay. We demonstrate here that HFD induces a severe oxidative stress in skeletal muscle especially in mitochondria, while curcumin administration reverses this deleterious effect. The regression analysis on the AUC data of IPGTT and MDA level has further ascertained the relationship of muscle mitochondrial redox status with insulin sensitivity. We hence suggest that muscular mitochondria are initial and important working sites for curcumin in attenuating oxidative stress and improving glucose tolerance. Detailed mechanisms underlying mitochondrial redox status in regulating insulin action are currently unclear. One possibility is that ROS impairs mitochondrial function, followed by reduced lipid oxidation and increased lipid accumulation in muscle. The elevation of muscle lipid content then activates protein kinase C, which blocks insulin receptor substrate-1 phosphorylation and downstream insulin signaling[29]. Further experiments are needed to assess whether curcumin is able to improve insulin signaling *via* blocking this pathological signaling pathway.

Redox balance is determined by both ROS production and anti-oxidative function (ROS elimination). Recent studies have indicated that an inability of the Nrf2 system in the elimination of ROS leads to the development of insulin resistance, whereas pharmacological or genetic activation of Nrf2 can improve insulin signaling, along with improved glucose metabolism[19,30]. To further investigate the underlying mechanism of curcumin on regulating redox balance, we examined Nrf2 signaling in insulin sensitive tissues. We found that HFD repressed Nrf2 function in muscles by reducing both total Nrf2 content and its nuclear portion, whereas curcumin markedly reversed these defects. Furthermore, curcumin also increased the expression of HO-1, a downstream target of Nrf2 and an essential anti-oxidative enzyme. These data are consistent with the reversal of oxidative stress in muscle but not in fat tissue or the liver. Therefore, these results suggest that curcumin-induced Nrf2 action is a novel mechanism against HFD-induced oxidative stress and to attenuate glucose intolerance. In line with the positive regulation of the Nrf2/HO-1 system on insulin sensitivity, HO-1 has been shown to repress oxidative stress and inflammation[31], and the administration of HO-1 inducer improves insulin sensitivity[32,33]. Due to the tight link of oxidative stress with inflammation[31], further studies should be conducted to address whether the activation of Nrf2 by curcumin is related to its anti-inflammatory effect. It should also be pointed out that although 15 d curcumin supplementation did not reduce the BW in HFD-fed animals, we began to see the effect of curcumin on reducing fat mass. We hence cannot rule out the possibility that curcumin-evoked Nrf2 signaling modulate lipid metabolism, adipogenesis, adipocyte differentiation, indirectly affecting glucose metabolism[17-19].

Curry containing curcumin, a popular natural spice in India, has been used as a traditional drug to treat inflammation, whereas modern studies further demonstrate the potential of curcumin to treat other major life-threatening diseases, such as heart failure, neurodegenerative diseases and diabetes[13,17,18,34]. The relative high safety and low cost of curcumin would also encourage its therapeutic applications in the future. In addition to the preventive effect of curcumin on insulin resistance, we demonstrated here that after the induction of obesity by HFD, short-term curcumin gavage still reversed glucose intolerance, clearly suggesting the therapeutic application of curcumin in the treatment of insulin resistance-related metabolic disorders. In light of its clinical usage, studies have been conducted and verified the effectiveness of curcumin in treating hyperglycemia-induced oxidative stress and related disorders, such as diabetic nephropathy[35,36]. In accordance, the present study provides a significant mechanism of Nrf2 activation by which curcumin can defend oxidative stress and mitochondrial redox imbalance and attenuate the abnormality of glucose metabolism in the condition of nutritional oversupply. Together these findings would place Nrf2 and curcumin as the new therapeutic targets and approaches for the treatment of hyperglycemia, as well as oxidative stress-related diseases.

COMMENTS

Background

Curcumin intervention attenuates insulin resistance and improves glucose disposal in various rodent models of insulin resistance and diabetes. The current mechanistic understanding on these beneficial effects is limited although curcumin has been shown to attenuate body weight (BW) gain and inflammatory response during high fat diet consumption. Whether curcumin improves insulin signaling *via* activating the endogenous nuclear factor erythroid-2-related factor-2 (Nrf2) anti-oxidative stress system and whether the improvement involves the attenuation of mitochondrial oxidative stress are unknown.

Research frontiers

The use of a naturally occurring compound in intervention is an optimum approach for the treatment and prevention of diabetes and obesity. Mitochondria are a primary site of production of free radicals. Extensive recent studies have revealed the fundamental importance of mitochondrial oxidative stress in the etiology of insulin resistance. The activation of the major anti-oxidative stress system Nrf2 was shown to improve insulin signaling and glucose disposal *in vivo*. Curcumin is able to activate Nrf2 signaling *in vitro*. Here the authors addressed the question whether curcumin, a naturally-occurring compound, improves insulin signaling *via* attenuating oxidative stress in mitochondria, *via* activating the Nrf2 signaling pathway.

Innovations and breakthroughs

The study showed here for the first time the stimulatory effect of curcumin in activating endogenous Nrf2 system *in vivo*. This finding will initiate further investigations on mechanistic exploration of the stimulation of Nrf2 system by naturally-occurring plant compounds.

The authors demonstrated here that curcumin improved insulin signaling and glucose disposal, associated with the attenuation of muscle mitochondrial oxidative stress. This finding defined muscle mitochondria as novel targets for curcumin.

The improvement observed in this animal model is independent of the BW lowering effect of curcumin. The authors have hence not only deepened the mechanistic understanding of the therapeutic effect of curcumin, but also revealed that attenuation of endogenous anti-oxidative stress is an important pathological event of high fat diet consumption.

Applications

This study identified a new target for curcumin and other naturally-occurring compounds in improving insulin sensitivity. It further supports the hypothesis that curcumin can be utilized in the treatment and prevention of diabetes and other metabolic disorders that involve insulin resistance.

Terminology

Insulin resistance is the pathophysiological condition that insulin can not exert normal function on its targeting tissues or cells, including muscle, liver and adipose tissue, or a higher concentration of insulin is required to evoke insulin signaling and glucose uptake. The abnormality is the basis for the development of insulin-resistant diseases, including type 2 diabetes mellitus, hypertension and certain cardiovascular diseases. Nrf2 anti-oxidative system: in the condition of oxidative stress, cytosol Nrf2 will be translocated into the nucleus. It will then bind to a battery of specific target genes in their promoter regions, stimulating the transcription of these genes. Nrf2 target genes encode a series of anti-oxidant enzymes, including HO-1.

Peer review

The present study showed that short term treatment with curcumin activates Nrf2 signaling and lowers blood glucose levels and oxidative stress in C57BL/6J mice fed on high-fat diet. It has two strengths. Firstly, demonstration of Nrf2 activation and nuclear translocation following curcumin treatment in an animal model of high-fat diet induced obesity and insulin resistance. Secondly, demonstration of reversal of high fat diet-induced malondialdehyde elevations in serum, muscle and muscle mitochondria.

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BRIEF ARTICLE

Lipoprotein(a) in type 2 diabetic subjects and its relationship to diabetic microvascular complications

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Abstract

AIM: To estimate the level of serum lipoprotein (a) [Lp (a)] in type 2 diabetes mellitus patients and to determine the relationship between Lp(a) in type 2 diabetes mellitus patients and micro-vascular complications.

METHODS: A cross sectional study was performed that enrolled 144 subjects with type 2 diabetes mellitus above the age of 25 years attending outpatient clinic of Government Medical College, Kozhikode. Lp(a) levels were measured quantitatively in venous samples using Turbidimetric Immunoassay in all subjects. Each patient was evaluated for micro vascular complications, namely diabetic retinopathy, nephropathy and neuropathy. The relationship between Lp(a) levels and the micro vascular complications was assessed by univariate analysis.

RESULTS: Mean age of cases was 53.93 ± 10.74 years with a male to female ratio of 1.3:1. Mean duration of diabetes was 9.53 ± 7.3 years. Abnormal Lp(a) levels (≥ 30 mg/dL) were observed in 38 (26.4%) diabetic subjects. Seventy-eight (54.16%) cases had diabetic nephropathy and significantly higher Lp(a) levels were found among these cases [Median 28.2 mg/dL (Interquartile range; IQR 24.4-33.5) *vs* 19.3 mg/dL (IQR 14.7-23.5); $P < 0.05$]. Retinopathy was present among 66 (45.13%) cases and peripheral neuropathy was detected among 54 (37.5%) cases. However, Lp(a) levels were not significantly different among those with or without retinopathy and neuropathy. Positive correlation was found between higher Lp(a) levels and duration of diabetes ($r = 0.165$, $P < 0.05$) but not with HbA1c values ($r = -0.083$).

CONCLUSION: Abnormal Lp(a) levels were found among 26.4% of diabetic subjects. Patients with diabetic nephropathy had higher Lp(a) levels. No association was found between Lp(a) levels and diabetic retinopathy or neuropathy. Longer duration of diabetes correlated with higher Lp(a) levels.

Key words: Diabetes mellitus; Lipoprotein(a); Micro vascular complications; Diabetic nephropathy; Diabetic retinopathy; Diabetic neuropathy

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INTRODUCTION

There has been a rising epidemic of diabetes mellitus in India in recent years and an alarming increase in the rate

of mortality and morbidity due to coexisting dyslipidemia, atherosclerosis and coronary artery disease. Diabetic micro vascular complications have become a major cause of chronic kidney disease, blindness and diabetic foot problems, which are preventable to some extent. Many risk factors, like the duration of diabetes, degree of glycemic control and age of the patient, are identified in causation of diabetic micro vascular complications.

Lipoprotein(a) [Lp(a)] is a low density lipoprotein-like particle containing Apo-lipoprotein B100 disulphide, linked to one large glycoprotein called Apo-Lp(a), a particle comprised of low density lipoprotein and covalently bound Apo-Lp(a), and is considered a pro-atherogenic, pro-thrombotic risk factor for coronary heart disease (CHD)[1]. Many prospective epidemiological studies have reported positive associations of baseline Lp(a) concentration with CHD risk[2-6].

There are conflicting reports on the relationship between Lp(a) levels and type 2 diabetes. Hyperinsulinemia tends to decrease Lp(a) levels among patients with type 2 diabetes[7,8] and some studies even showed an inverse relationship between Lp(a) levels and incident type 2 diabetes[9,10]. However, some Asian studies showed a strong association between type 2 diabetes and elevated Lp(a) levels[11,12]. Similarly there are conflicting reports on the evidence of association between Lp(a) levels and diabetic micro vascular complications like nephropathy, retinopathy and neuropathy[13-20].

There is insufficient data from the Indian subcontinent on Lp(a) levels and its role in micro vascular complications among patients with type 2 diabetes mellitus. The purpose of the present study was to estimate the serum Lp(a) levels in type 2 diabetic patients and to determine if there is any relationship between serum Lp(a) levels and diabetic micro vascular complications.

MATERIALS AND METHODS

The study included patients with type 2 diabetes mellitus above the age of 25 years who were attending the medical and diabetic outpatient clinics of Government Medical College, Kozhikode, a tertiary-care teaching hospital in northern Kerala, South India. This study was planned with the following aims: (1) to estimate the level of serum Lp(a) in type 2 diabetes mellitus patients; and (2) to determine the relationship between Lp(a) levels and diabetic micro vascular complications. The exclusion criteria were: (1) patients who were already on lipid lowering drugs or glitazones and females taking oral contraceptive pills or hormone replacement therapy; (2) familial hypercholesterolemias; (3) hypothyroidism, including subclinical hypothyroidism (with thyroid stimulating hormone values above 5.5 μIU/mL); (4) those who are seriously ill and/or requiring hospitalization or with chronic liver or kidney disease with serum creatinine ⩾ 2 mg%; and (5) those who were in the habit of alcohol use.

Subjects who were taking medications for hyperlipidemia or medications known to affect the lipid profile were excluded. Subjects with familial hyperlipidemia, pregnancy, hypothyroidism, alcoholism, as well as those with signs and/or symptoms of active infection or stressful conditions were excluded as they are known to alter the Lp(a) levels.

This was a cross sectional study. Informed consent was obtained from each of the participants and the study was approved by the Institutional Review Board. A detailed history including dietetic history was taken. Physical examination included height, weight and body mass index (BMI). BMI was calculated by determining weight in kilograms and dividing by the height in meters squared. Waist circumference was measured using a measuring tape in centimeters at the point where the mid axillary line touches the highest point of iliac crest. The plane of the tape was held parallel to the floor with the tape snug but without compression of the skin. The measurement was made at a normal minimal respiration.

Neuropathy assessment was done in both feet with vibration perception using a tuning fork of 128 Hz, elicitation of ankle jerks and testing with monofilament of 5.07 size (thickness), equivalent of 10 gm of linear force. If any two of the three tests were positive, the patient was considered to have neuropathy after excluding other causes for neuropathy with a reasonable clinical and appropriate laboratory evaluation. Examination of the retina was done through dilated pupils to determine the level of non-proliferative diabetic retinopathy, proliferative diabetic retinopathy (PDR) and macular edema by a qualified ophthalmologist. The definitions were based on the International Classification of Diabetic Retinopathy. Screening for microalbuminuria can be performed by measurement of the albumin-creatinine ratio in a random, spot collection (preferred method); the analysis of a spot sample for the albumin-creatinine ratio is strongly recommended by most authorities. In the present study, two of three specimens collected within a 3 to 6 mo period were used for quantification to include in the respective group.

A venous blood sample was collected after a 12 h overnight fasting for estimation of Lp(a) levels. The measurement is performed with the person in a baseline stable condition. Lp(a) level was measured by turbidimetric immunoassay. The reference value for Lp(a) level in the normal population is < 30 mg/dL. HbA1c was estimated using high performance liquid chromatography method. Other laboratory investigations, including fasting and post prandial blood sugars, blood urea, serum creatinine and thyroid stimulating hormone, were done in all the patients.

Statistical analysis

Data are reported as median and inter quartile range (IQR) or mean ± SD for continuous variables and as proportions for categorical variables. Continuous variables were analyzed by *t*-test and Pearson's correlation when data was normally distributed and by Mann Whitney *U* test when data was not normally distributed. A *P* value

Table 1 Lipoprotein(a) levels among patients with or without retinopathy and neuropathy

	No. of cases	Lp(a) level (mg/dL)	
		Median	Inter quartile range
Retinopathy present	66	24.8	16.1-29.4
Retinopathy absent	78	22.9	14.9-27.6
Neuropathy present	54	23.2	18.1-27.3
Neuropathy absent	90	24.6	17.6-28.1

Lp(a): Lipoprotein(a).

Table 2 Definitions of abnormalities in albumin excretion and lipoprotein(a) levels with albumin-creatinine ratio

Albumin/creatinine ratio (μgm/mg creatinine)	No. of cases	Lp(a) levels (mg/dL)	
		Median	Inter quartile range
Normal (< 30)	66	19.3	14.7-23.5
Micro (30-299)[a,b]	58	26.4	20.2-32.8
Macroalbuminuria (≥ 300)[a]	20	33.2	30.3-36.1

[a]$P < 0.05$ *vs* normal; [b]$P < 0.05$ *vs* macroalbuminuria. **Lp(a): Lipoprotein(a).**

< 0.05 was considered to indicate statistical significance. Statistical analysis was done using SPSS version 13.0 for Windows.

RESULTS

A total of 144 subjects satisfying the inclusion criteria were included in the study. The mean age was 53.93 ± 10.74 years and the male to female ratio was 1.3:1. Mean duration of diabetes was 9.53 ± 7.3 years. Mean BMI was 25.16 ± 3.9 kg/m^2 with a waist circumference of 91.94 ± 8.8 cm. Mean systolic blood pressure was 134.12 ± 17.1 mmHg and mean diastolic blood pressure was 83.12 ± 9.2 mmHg. Mean HbA1c was 8.01% ± 2.15%. With regard to current diabetic management: 3% of patients were on diet alone; 70% were on oral antidiabetic drugs like metformin and/or sulfonylurea; 19% were on oral antidiabetic drugs (metformin and/or sulfonylurea) and insulin; and 8% were on insulin alone (Patients on glitazones were not included in the present study).

Lp(a) level was done in all 144 subjects (normal range in serum is up to 30 mg/dL). Lp(a) levels were abnormal in 38 (26.4%) cases and normal in 106 (73.6%) cases. Higher Lp(a) levels had a significant positive correlation to the duration of diabetes ($r = 0.165$; $P < 0.05$). However, Lp(a) levels did not have a correlation to HbA1c values ($r = -0.083$; P = insignificant).

Lp(a) levels and micro vascular complications

Retinopathy was assessed in all 144 patients. 78 (54.2%) did not have retinopathy. 66 (45.8%) cases had evidence of diabetic retinopathy, of whom 40 (27.8%) cases had mild non-proliferative retinopathy, 13 (9%) had moderate non-proliferative retinopathy and 8 (5.6%) had severe non-proliferative retinopathy. Five (3.4%) cases had PDR. There was no statistically significant difference in Lp(a) levels among patients with and without diabetic retinopathy (Table 1).

Diabetic neuropathy was present in 54 (37.5%) patients and absent in 90 patients (62.5%) but there was no statistically significant difference in Lp(a) levels among patients with and without diabetic neuropathy (Table 1).

Lp(a) levels and diabetic nephropathy

Seventy-eight (54.16%) cases had diabetic nephropathy (microalbuminuria or overt proteinuria). Median Lp(a) levels in this group was 28.2 mg/dL (IQR 24.4-33.5), whereas those without nephropathy had a median Lp(a) level of 19.3 mg/dL (IQR 14.7-23.5) and this difference was statistically significant ($P < 0.05$). Intergroup comparison of median Lp(a) levels between patients with microalbuminuria and macroalbuminuria also showed statistical significance (Table 2).

DISCUSSION

Diabetes mellitus confers a two-fold higher risk for a wide range of vascular diseases, independent of other conventional risk factors[21]. Any additional risk factor along with diabetes would increase the vascular risk that might prove to be catastrophic to the sufferer. High Lp(a) level has been proven to be a risk factor for atherosclerosis and related morbidity and mortality in many studies[2-6]. It would be logical to consider higher vascular risk among diabetic patients with elevated Lp(a) levels although such an association is yet to be proven in controlled trials.

Type 2 diabetics are usually hyperinsulinemic and insulin tends to lower the Lp(a) levels[7,8]. Large population-based studies have even shown an inverse association between Lp(a) levels and incident diabetes[9,10]. However, some Asian studies clearly showed higher Lp(a) levels among type 2 diabetics[8,11,12]. These conflicting reports on the association between Lp(a) levels and type 2 diabetes prompted us to estimate the Lp(a) levels in this diabetic cohort.

A significant proportion of type 2 diabetics (26.4%) had elevated Lp(a) levels, as observed by other workers[8,11,12]. Higher Lp(a) levels were observed among those with a longer duration of diabetes in this study, similar to the observations made by Habib *et al*[8]. Higher Lp(a) levels among patients with a longer duration of diabetes may be related to lower plasma insulin levels in such individuals. Because vascular risk is directly related to the duration of diabetes, the possible contribution of elevated Lp(a) levels to higher vascular risk among type 2 diabetics demands investigation in future clinical trials. A cross sectional analyses of two community-based studies showed that Lp(a) is a strong independent predictor of CHD risk in type-2 diabetic women, but not in men or in men or women without type-2 diabetes[22]. Already there is some evidence showing a strong association between peripheral occlusive arterial disease (a marker of systemic

atherosclerosis) and serum Lp(a) levels in patients with diabetes[23]. The present study did not show any relationship of Lp(a) levels to glycemic control, as in one previous study[24].

The present study showed a statistically significant association between higher Lp(a) levels and diabetic nephropathy (both microalbuminuria and overt proteinuria). Tseng[14] from Taiwan also recently observed high Lp(a) levels among type 2 diabetic patients with overt proteinuria although an earlier study[13] did not show such an association. Our observation of high Lp(a) levels among those with overt proteinuria in the present study has important clinical implications as Lp(a) level is an independent risk factor for the progression of diabetic nephropathy in type 2 diabetic patients with overt proteinuria, as shown by Song *et al*[25].

We did not observe any statistically significant association between Lp(a) levels and diabetic retinopathy in this cohort. Some previous studies have shown an association between Lp(a) levels and retinopathy[15,16], while others have not[17,18]. Similar to the observations made by earlier workers[19,20], we were also unable to find any association between diabetic neuropathy Lp(a) levels.

The small number of subjects selected for evaluation of a common clinical problem like type 2 diabetes mellitus is an important limitation of this study. However, the observation of high Lp(a) levels in a significant proportion of cases and the association between Lp(a) levels and diabetic nephropathy were especially noteworthy. Larger studies are necessary to elucidate the vascular risk related to Lp(a) levels in Indian patients with type 2 diabetes for strategic planning of preventive measures.

In conclusion, Lp(a) levels were abnormal in 26.4% of type 2 diabetic patients in the present study. A significantly higher proportion of patients with diabetic nephropathy had higher Lp(a) levels compared to those without nephropathy. Lp(a) levels were comparable among patients with or without diabetic retinopathy and diabetic peripheral neuropathy. A longer duration of diabetes had a positive correlation with higher Lp(a) levels. However, higher HbA1C levels did not have any correlation with Lp(a) levels.

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COMMENTS

Background

There has been a rising epidemic of diabetes mellitus in India in recent years. Diabetic micro vascular complications have become a major cause for chronic kidney disease, blindness and diabetic foot problems, which are preventable to some extent. Many risk factors, like the duration of diabetes, degree of glycemic control and age of the patient, are identified in the causation of diabetic micro vascular complications. There are conflicting reports on the evidence of the association between lipoprotein(a) [Lp(a)] levels and diabetic micro vascular complications like nephropathy, retinopathy and neuropathy. The purpose of the present study was to estimate the serum Lp(a) levels in type 2 diabetic patients and to determine if there is any relationship between serum Lp(a) levels and diabetic micro vascular complications.

Research frontiers

High Lp(a) levels among those with overt proteinuria in the present study has important clinical implications as Lp(a) level is an independent risk factor for the progression of diabetic nephropathy in type 2 diabetic patients with overt proteinuria. Larger studies are necessary to elucidate the vascular risk related to Lp(a) levels in Indian patients with type 2 diabetes for strategic planning of preventive measures. Increased concentrations of Lp(a) lipoprotein might partly explain the increased morbidity and mortality from cardiovascular disease observed among patients with diabetic nephropathy and Lp(a)-lowering therapy might offer benefits in subgroups of patients with high Lp(a) levels.

Innovations and breakthroughs

Lp (a) level is an independent risk factor for the progression of diabetic nephropathy in type 2 diabetic patients with overt proteinuria, as shown by Song *et al*. The observation of high Lp(a) levels among those with overt proteinuria in the present study, as shown in some previous studies, has important clinical implications. This may help to identify the high risk group to implement intensive follow up.

Applications

This study suggests that Lp(a) may be an independent risk factor for the progression of diabetic retinopathy, apart from other known risk factors like the duration of diabetes, degree of glycemic control and age of the patient.

Terminology

Lp(a) is a low density lipoprotein-like particle containing Apo-lipoprotein B100 disulphide, linked to one large glycoprotein called Apo-Lp(a).

Peer review

The study offers an interesting insight into the correlation between Lp(a) levels and proteinuria in type 2 diabetes mellitus and progression of nephropathy.

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Events Calendar 2012

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Santa Fe, NM, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

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5th International Conference on Advanced Technologies and Treatments for Diabetes
Barcelona, Spain

February 9-10, 2012
EC Conference on Diabetes and Obesity Research - Save the Date
Brussels, Belgium

February 21, 2012
Association of Children's Diabetes Clinicians 6th Annual Meeting
Coventry, United Kingdom

February 23, 2012
Diabetes and kidney disease: advances and controversies
Birmingham, United Kingdom

March 1-3, 2012
International conference on Nutrition and Growth
Paris, France

March 7-9, 2012
Diabetes UK Annual Professional Conference 2012
Glasgow, United Kingdom

March 15 -16, 2012
Monogenic Disorders of Insulin Secretion: Congenital Hyperinsulinism and Neonatal Diabetes
Philadelphia, PA, United States

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Hollywood, CA, United States

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Harrogate, United Kingdom

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Rio de Janeiro, Brazil

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Riga, Latvia

March 29-April 1, 2012
New Frontiers in Diabetes Management
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Palm Coast, FL, United States

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INSTRUCTIONS TO AUTHORS

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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]

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

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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

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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

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