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Forum Review

Redox Modulation of Insulin Signaling and Endothelial Function

RAYMOND CHRISTON,[1] OLIVIER DROUIN,[2] and ANDRE MARETTE[2]

ABSTRACT

Reactive oxygen and nitrogen species (ROS and RNS) recently emerged as critical signaling molecules in cardiovascular research. Several studies over the past decade have shown that physiological effects of vasoactive factors are mediated by these reactive species and, conversely, that altered redox mechanisms are implicated in the occurrence of metabolic and cardiovascular diseases. Oxidant stress occurs when ROS and/or RNS production exceeds the cell natural antioxidant systems, and pathological events ensue. Cardiovascular risk factors are associated with an imbalance of the redox equilibrium toward oxidative stress, leading to endothelial activation and proinflammatory processes implicated in atherogenesis and metabolic disorders. Recent studies indicate that insulin and insulin-sensitizing drugs activate antiinflammatory pathways that may limit oxidant stress in insulin target tissues. The main goal of this brief review is to discuss recent progress in the field of cellular redox signaling as it pertains to insulin modulation of vascular endothelial function in cardiovascular diseases. *Antioxid. Redox Signal.* 7, 1062–1070.

INTRODUCTION

IT IS NOW WELL ESTABLISHED that the metabolic and growth-promoting actions of insulin are modulated by oxidative stress and antioxidant mechanisms. The cellular redox status is a key determinant of insulin action, and there is now a large body of evidence to suggest an important role of oxidative stress in mediating both insulin resistance and the vascular complications associated with diabetes. Multiple hypotheses have been put forward regarding the role of insulin action on vascular tissues as related to glucose homeostasis, control of blood pressure and blood flow, and development of vascular complications. In the present article, we will briefly review current knowledge on the mechanisms by which redox mechanisms modulate insulin signaling. Particular emphasis will be placed on the role of oxidative stress in promoting insulin resistance and endothelium activation, leading to vascular dysfunction. The key role of dietary fatty acids in modulating insulin signaling and endothelial activation will also be reviewed.

INSULIN SIGNALING IN THE ENDOTHELIUM

Insulin has direct effects on the vascular endothelium, consistent with the identification of insulin receptors (IRs) in endothelial cells of both large and small blood vessels (9, 92). Acute insulin infusion induces vasodilatation (increased leg blood flow) in humans (11, 12), and this effect is reduced in subjects with impaired glucose tolerance and diabetes (79). The stimulatory effect of insulin on blood flow has been reported to be dependent on the release of nitric oxide (NO) from endothelial NO synthase (eNOS) (78). The vasodilatory effect of insulin can be acute (within minutes), but also increases with time (2–8 h) through enhanced expression of eNOS, a mechanism that is also defective in obesity-linked diabetes (51). The vascular action of insulin is believed to amplify the hormone action to enhance glucose uptake by increasing the delivery of glucose and insulin to the capillary bed. The implication of eNOS in insulin-mediated glucose disposal is also supported by the observation that *in vivo* infu-

[1]Lipides Membranaires et Fonctions Cardiovasculaires, UMR INRA-Université Paris XI, Faculté de Pharmacie, Châtenay-Malabry, and [2]Department of Anatomy and Physiology and Lipid Research Unit, Laval University Hospital Research Center, Ste-Foy, Québec, Canada.

sion of the NO synthase (NOS) inhibitor L-NAME (N^{ω}-nitro-L-arginine methyl ester) significantly reduced insulin-stimulated glucose uptake by muscles (69) and by the finding that mice with a targeted disruption in eNOS develop insulin resistance (75).

Insulin exerts its metabolic and vascular actions by a complex signaling cascade (see Fig. 1). Following binding of insulin to its receptor α-subunits, autophosphorylation of the transmembrane β-subunits occurs, leading to intrinsic activation of the receptor tyrosine kinase activity. The activated IR increases the tyrosine phosphorylation of IR substrates 1 and 2 (IRS-1, IRS-2), which serve as docking sites for multiple downstream insulin effectors. Activation of the IR/IRS pathway has been demonstrated in both endothelial cells (91) and microvessels (45). Evidence for a key role of the IR and IRS-1/2 in mediating the endothelial action of insulin has been also obtained by the creation of knockout mouse models for these proteins. Thus, IRS-1 knockout mice not only showed metabolic disturbances, but also exhibited impaired endothelium-dependent vascular relaxation (1). On the other hand, lack of IRS-2 in transgenic mice renders the vasculature more susceptible to injury in insulin-resistant states (52). Furthermore, mice lacking IR specifically in vascular endothelial cells have reduced expression of eNOS and exhibited altered insulin sensitivity to dietary salt intake (90).

A key downstream element in the stimulation of eNOS by insulin is phosphatidylinositol (PI) 3-kinase. The p85 regulatory subunit of PI 3-kinase binds to tyrosine-phosphorylated IRS proteins, leading to activation of the 110-kDa catalytic (p110) subunit. Both inhibition of PI 3-kinase by wortmannin and overexpression of a mutated p85 adapter subunit lacking inter Src homology 2 domain required for binding to the p110 catalytic subunit significantly inhibit insulin-mediated eNOS activation by insulin (96, 97). Activation of PI 3-kinase catalyzes the formation of phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$), which is required for the activation of 3′-phosphoinositide-dependent protein kinases (PDK-1/2) and phosphorylation of the serine/threonine kinase Akt by dual phosphorylation on Thr^{308} and Ser^{473}. Quon and colleagues (63) recently reported that overexpression of wild-type PDK-

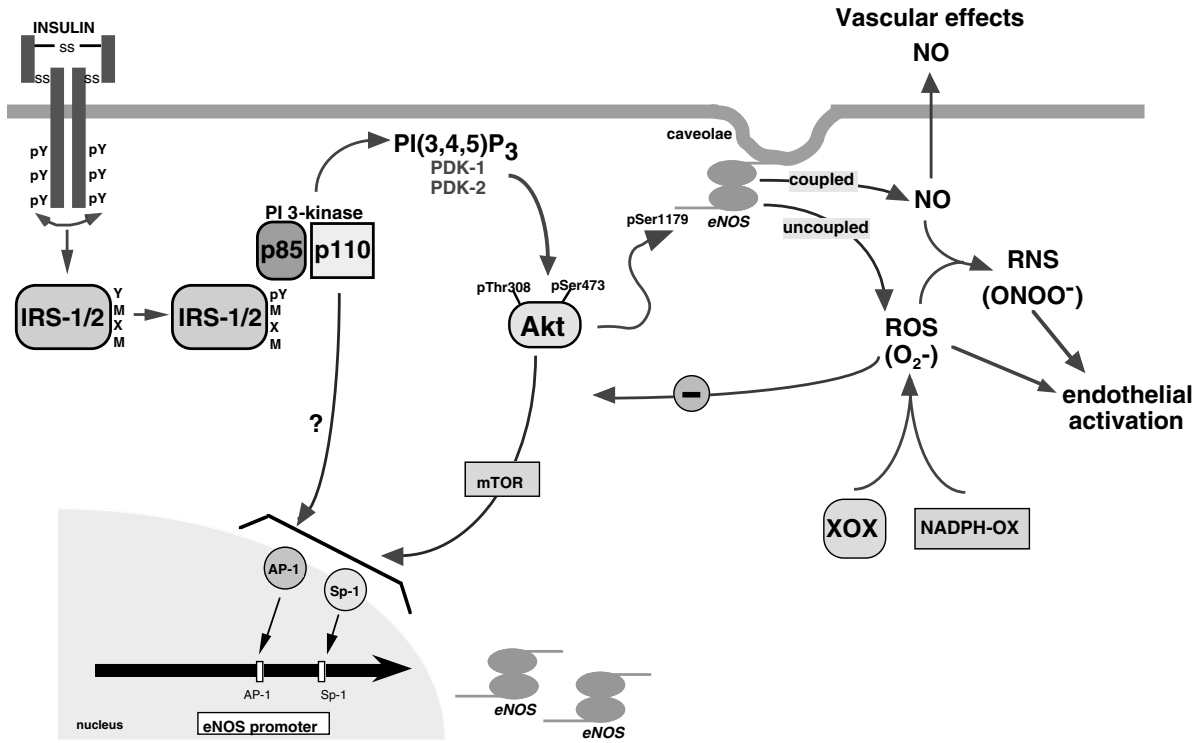

FIG. 1. Insulin signaling to eNOS activation and putative role of ROS and RNS in endothelial activation and impaired insulin-mediated eNOS regulation. Insulin stimulates eNOS activity in endothelial cells by binding its receptor α-subunits leading to the autophosphorylation of the transmembrane β-subunits and intrinsic activation of the receptor tyrosine kinase activity. The activated IR phosphorylates IRS-1 and IRS-2 on tyrosine residues. Tyrosine-phosphorylated IRS proteins serve as docking proteins for the p85 regulatory subunit of the PI 3-kinase, which enables activation of its p110 catalytic subunit and production of $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ production is essential for activation of PDK-1 and the putative PDK-2, leading to the phosphorylation of Akt on Thr^{308} and Ser^{473}, respectively. Akt then phosphorylates eNOS on Ser^{1179}, and NO is generated (coupled condition). Long-term (>4 h) insulin stimulation also increases eNOS expression, by a PI 3-kinase/Akt/mammalian target of rapamycin (mTOR)-dependent mechanism involving the combined activation of AP-1 and Sp-1 transcription factors. In pathological conditions associated with reduced bioavailability of the cofactor BH_4, eNOS can be "uncoupled" leading to eNOS-mediated O_2^- production. Other cellular sources of ROS (notably O_2^-) are xanthine oxidase (XOX) and NAD(P)H oxidase (NADPH-OX). Increased O_2^- production may lead to formation of RNS, such as $ONOO^-$. Both ROS and RNS are implicated in endothelial activation, a primary event in atherogenesis, and may also blunt insulin signaling to eNOS activation and thus impair endothelial regulation.

1 enhances insulin-stimulated production of NO, whereas a kinase-inactive mutant PDK-1 inhibits this effect of insulin, providing evidence that PDK-1 is a necessary component of the signaling cascade leading to eNOS activation by insulin. Interestingly, activation of eNOS by insulin is dependent on its phosphorylation on Ser^{1179} by Akt (Fig. 1), but independent of the classical calcium-dependent pathway (62). This calcium-independent activation of eNOS by insulin appears to involve formation of a complex between calmodulin-bound eNOS, Akt, and heat shock protein 90 (81). The long-term vasodilatory effect of insulin, mediated by increased eNOS expression, is also mediated via activation of the PI-3 kinase pathway (51), and through the action of the transcriptional factors activator protein-1 (AP-1) and Sp-1 (31).

Insulin-mediated Akt activation has also been shown to play an important role in the prevention of endothelial cell apoptosis, which may importantly contribute to cell homeostasis and the integrity of the endothelium. In endothelial cells, Akt seems to mediate its antiapoptotic effect, at least in part, via phosphorylation of caspase-9 (40).

VASCULAR OXIDATIVE STRESS, INSULIN ACTION, AND ENDOTHELIAL FUNCTION

The vascular endothelium is a metabolically responsive tissue where NO is arguably the most important factor responsible for endothelium-dependent vascular relaxation. NO not only is involved in relaxation of vascular smooth muscle, but also mediates inhibition of platelet adhesion and aggregation, prevention of vascular smooth muscle proliferation, and adhesion of leukocytes to the endothelium (see 70). Conversely, in endothelial dysfunction, oxidant stress and associated alterations in the local redox state elicit a number of molecular alterations that produce increased monocyte adhesiveness and permeability to plasma lipoproteins. The vasodilatory effects of endogenously produced NO are overwhelmed by a preponderance of cellular oxidants [reactive oxygen species (ROS)] and other vasoconstricting substances causing endothelial dysfunction in multiple metabolic/vascular diseases, including diabetes and atherosclerosis (87).

ROS are often considered as pathogenic factors, but in fact play an important role in normal vascular homeostasis and function (43). Endothelial function is regulated by a dynamic balance between NO and other oxidants such as superoxide (O_2^-) and peroxide (H_2O_2). NO acts as an antioxidant by scavenging O_2^- and exerts antiinflammatory actions by reducing endothelial inflammation, at least in part by inhibition of nuclear transcription factors such as nuclear factor-κB (NF-κB), reduction of adhesion molecules, and cytokine expression (44). Endogenous antioxidant defense mechanisms, such as superoxide dismutase, glutathione peroxidase, chain terminators (vitamins A, E, and C), hemoglobin, and catalase, are also thought to be important in counterbalancing oxidant stress mechanisms (8, 30, 57). However, when oxidation products increase above the buffering capacity of NO and antioxidant enzymes, potent oxidants are generated. In addition, exaggerated and sustained production of NO following activation of inducible NOS (iNOS), a cytokine-inducible NOS isoform, will promote NO reaction with O_2^- to produce the potent oxidant peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS) (77). ROS and RNS cause induction of adhesion molecules on the surface of endothelial cells, a mechanism dependent on transcriptional activation by NF-κB and AP-1 (38, 50). Monocytes are a cellular source of ROS, particularly O_2^-, which has been proposed to be involved in the activation of transcription factors and induction of adhesion molecules in the endothelial cells (67, 68). Experimental studies using antioxidants confirmed that the noxiousness of oxidized lipoproteins was due to the oxidation products they contain, mainly oxysterols, fatty acids-derived oxidation products, lysophosphatidylcholines, and isoprostanes (60, 66, 95). Furthermore, it has been shown that $ONOO^-$ stimulates the formation of isoprostanes *in vitro* and that NO inhibited this effect (54).

Endothelial dysfunction is clearly associated with insulin resistance in chronic metabolic diseases, such as obesity-linked diabetes and atherosclerosis. The vasodilatory effect of insulin is markedly impaired in these diseased states (21, 79, 83). However, it is still unclear whether the defective vascular action of insulin is linked to oxidative stress. Few studies have carefully investigated the role of ROS in impairing insulin signaling to eNOS activation in vascular tissues of insulin-resistant subjects or animal models. H_2O_2 has been reported to inhibit insulin signaling to Akt in smooth muscle vascular cells (33). Moreover, oxidative stress has been shown to cause impairment in insulin-induced PI 3-kinase and Akt activation in adipocytes, an effect that is reversed by the antioxidant lipoic acid (71–73, 84, 85). However, the potential effect of ROS on endothelial insulin signaling remains to be investigated. On the other hand, a growing number of studies indicate that oxidative stress reduces endothelium NO bioavailability, most probably consequent to ROS production by NAD(P)H oxidase, xanthine oxidase, or eNOS itself (see Fig. 1). Indeed, it has been shown that, in insulin-resistant states, eNOS generates less NO but more O_2^- due to enzymatic "uncoupling" of eNOS (24). This is likely the consequence of decreased availability of tetrahydrobiopterin (BH_4), an essential cofactor for eNOS (23, 89). Such a role for BH_4 is supported by the observations that endothelial dysfunction is improved by high-concentration BH_4 supplementation in vessel rings from diabetic or atherogenic animals (41, 55, 76). Furthermore, BH_4 administration acutely improves NO-mediated effects on forearm blood flow in diabetic (37) or hypercholesterolemic subjects (80). The key role of BH_4 in the regulation of eNOS by vascular oxidative stress was recently demonstrated in a transgenic mouse model with endothelial-targeted overexpression of the rate-limiting enzyme in BH_4 synthesis, guanosine triphosphate–cyclohydrolase I (GTPCH). It was found that GTPCH overexpression in the endothelium selectively augments endothelial BH_4 levels in both insulin-deficient diabetic mice and atherosclerosis-prone apolipoprotein E-knockout mice, which was associated with protection from endothelial O_2^- production and restoration of NO-mediated vasodilatation (5, 6).

Current treatment strategies for vascular disease aim to improve endothelial function either through their ability to alleviate oxidant stress, or by enhancing NO production or

bioavailability. These treatments include the use of antioxidants and angiotensin-converting enzyme inhibitors, as well as drugs that predominantly act via lipid-lowering mechanisms (30). Interestingly, insulin-sensitizing drugs are also gaining further interest in the treatment of vascular diseases because they can limit hyperglycemia-mediated oxidative damage, but also mimic the antiinflammatory properties of insulin. Indeed, insulin has a potent antiinflammatory effect that has been shown *in vitro* in human aortic endothelial cells and in mononuclear cells (2, 4), both of which initiate atherosclerotic inflammation (27). Insulin was shown to suppress the transcription factor NF-κB and the expression of adhesion molecules [*e.g.,* intracellular adhesion molecule-1 (ICAM-1)], as well as the chemoattractant protein, monocyte chemotactic protein-1 (MCP-1), possibly linked to its ability to stimulate the release of NO from endothelial cells and to enhance the expression of eNOS. In obese subjects, a low dose of insulin led to the suppression of intranuclear NF-κB, a decrease in NADPH oxidase activity and O_2^- production, and decreased C-reactive protein, ICAM-1, and MCP-1 (10). These authors also observed an increased cellular IκB, which binds NF-κB and prevents its translocation into the nucleus, thus inhibiting the transcriptional action of NF-κB. The action of NF-κB is considered central to atherosclerosis because it induces the transcription of multiple proinflammatory molecules. Insulin also suppresses AP-1, a transcription factor involved in matrix metalloproteinase expression in the atherosclerotic plaque, which is thought to be involved in plaque rupture (32).

One class of insulin-sensitizing drugs that has received a lot of attention lately is the thiazolidinediones (TZDs). This class of molecules interacts with peroxisome proliferator-activated receptors (PPARs), which are nuclear receptors with key roles in the regulation of lipid and glucose metabolism. Ligands for PPARs, particularly PPARγ, promote insulin sensitization in obesity and have also been shown to produce selected antiinflammatory effects and to reduce the progression of atherosclerosis in animal models (see 61). This is consistent with the expression of PPARγ receptors in all cell types relevant to immune function and atherosclerosis, *i.e.,* monocytes, macrophages, vascular smooth muscle cells, and endothelial cells (15). The TZD troglitazone has been reported to improve endothelial function and arterial reactivity in altered metabolic states associated with vascular disorders (18, 36, 74, 82, 94), and there is evidence suggesting that these beneficial effects are explained, at least in part, by modulation of oxidative processes (7, 42). Treatment of obese insulin-resistant patients with troglitazone has been shown to suppress NF-κB levels and to reduce generation of ROS by NAD(P)H oxidase and lipid peroxidation (3, 34, 35). These antioxidant effects were observed independently from the classical lipid-lowering and hypoglycemic actions of the drug and may be independent of PPARγ binding.

Another insulin-sensitizing drug that has been reported to have antioxidant properties is the biguanide agent metformin. Metformin reduces lipid peroxidation while enhancing levels of erythrocyte superoxide dismutase and plasma glutathione. Metformin also improves both brachial artery endothelial dysfunction and insulin resistance in patients with non–insulin-dependent diabetes mellitus, benefits that were independent of a hypoglycemic effect (59). More recently, metformin was shown to scavenge hydroxyl (but not O_2^-) radical in human leukocytes (16), suggesting that ROS scavenging could contribute to the benefits of metformin treatment, especially those related to the improvement in the cardiovascular outcomes in diabetes.

The antiinflammatory effects of both PPARγ ligands and metformin may also be linked to their ability to increase the activity of AMP-activated protein kinase (AMPK), an energy-sensing enzyme that is activated in response to cellular stress. AMPK has recently emerged as an attractive novel target for the treatment of obesity and type 2 diabetes because its activation increases fatty acid oxidation and improves glucose homeostasis. However, we have recently reported (65) that pharmacological activation of AMPK by the insulin-sensitizing drugs troglitazone and metformin markedly inhibits inducible nitric oxide (NO) synthase (iNOS), a proinflammatory mediator in multiple chronic inflammatory states, including obesity-linked diabetes (13, 47–49, 58, 64) and atherosclerosis (14, 25). As depicted in Fig. 2, treatment of L6 myocytes with the AMPK activator AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) or the insulin-sensitizing

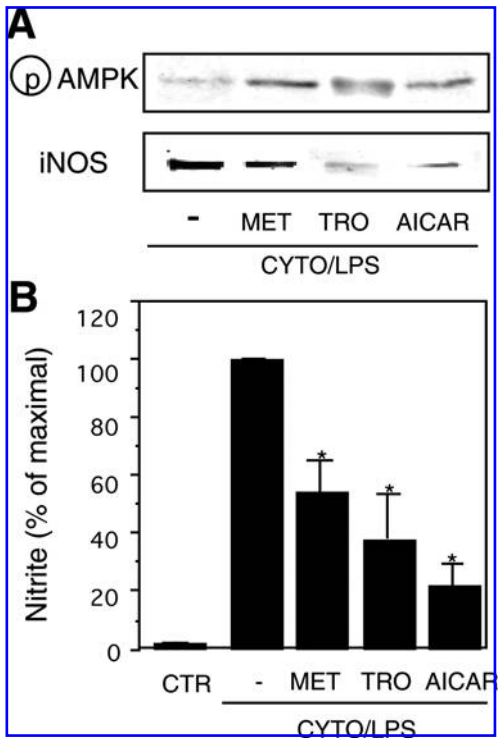

FIG. 2. AMPK activators inhibit iNOS induction in L6 myocytes. Muscle cells were treated for 24 h with or without cytokines (TNF-α, 10 ng/ml; and interferon-γ, 200 units/ml) and lipopolysaccharide (10 μg/ml) (CYTO/LPS) in the presence of AICAR (2.5 m*M*), metformin (MET; 2 m*M*), or troglitazone (TRO; 10 μ*M*). (**A**) Representative immunoblots of AMPK (Thr^{172}) phosphorylation and iNOS protein levels following treatment with AMPK activators. (**B**) NO production was measured by nitrite accumulation in the incubation medium. Results are expressed as means ± SE for three to six individual experiments. *$p < 0.05$ as compared with CYTO/LPS values.

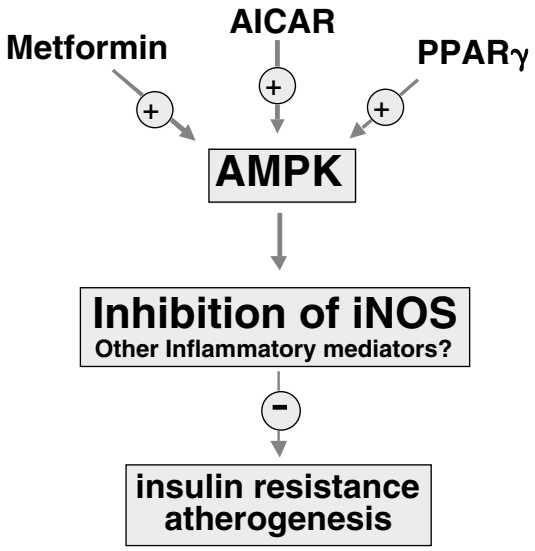

FIG. 3. Insulin-sensitizing drugs inhibit iNOS induction in insulin target cells: a new antiinflammatory mechanism. AMPK can be activated by insulin-sensitizing drugs such as metformin, PPARγ agonists (troglitazone, 15-Δ-prostaglandin J_2), and the AMPK activator AICAR. This leads to inhibition of iNOS protein expression in insulin target cells, such as myocytes, adipocytes, and macrophages. Whether this effect is also observed in endothelial cells remains to be validated. As iNOS is implicated in the pathogenesis of both insulin resistance and atherogenesis, AMPK may represent a novel therapeutic target for subjects with diabetes and cardiovascular diseases.

drugs metformin and troglitazone activates AMPK, as detected by its phosphorylation on Thr^{172}. This is associated with marked inhibition of iNOS protein expression and NO production in these cells. AMPK-mediated iNOS inhibition was also observed in adipocytes and macrophages and primarily resulted from posttranscriptional regulation of the iNOS protein (65). Our findings thus show for the first time that AMPK is a novel antiinflammatory signaling pathway and as such represents a promising therapeutic target for immune-inflammatory disorders (Fig. 3). More studies will be required to establish fully a role for the antiinflammatory properties of AMPK in the therapeutic approach to endothelial dysfunction in metabolic and vascular diseases.

REDOX MODULATION OF ENDOTHELIAL FUNCTION BY FATTY ACIDS

The increase in ROS and RNS is known to represent the main source of oxidative stress, *i.e.,* an imbalance between reactive molecular species and the cell antioxidant system. There is converging evidence that this cell status may be caused, at least in part, by a number of stimuli, including hyperglycemia and/or high free fatty acids level in circulation (28), advanced glycation end products (53), inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (39), or oxidized low-density lipoproteins (LDL) (22). The effect of fatty acids, particularly the polyunsaturated fatty acids (PUFA), on the vascular endothelium activation has received a lot of attention in recent years. Fatty acids have been shown to modulate the cellular oxidant/antioxidant equilibrium in liver cells, an effect that is fatty acid type-specific (19). Dietary n-6 PUFA may constitute a potential source of oxidative stress in pathological conditions and, without adequate protection by antioxidants, may affect NO metabolism in both animal models and subjects with coronary heart disease (46, 86, 88). The bioavailability of antioxidant systems is therefore of considerable importance to counterbalance these PUFA autooxidation processes.

In contrast to n-6 PUFA, n-3 PUFA in fish oils, as well as linoleic acid conjugates (CLA), appear to blunt vascular endothelial activation (56). These fatty acids could improve the cellular redox status and thus limit the impact of oxidant stress on endothelial function. As reviewed elsewhere (17, 20), several *in vitro* studies in the past few years have shown that fish oils enriched in docosahexaenoic acid and in eicosapentaenoic acid can reduce vascular cell adhesion molecule-1 expression and adhesion of monocytes to endothelial cells. Results obtained from *in vivo* studies, however, are less consistent, but some studies did observe improved endothelial function in subjects receiving a diet rich in n-3 PUFA (17). The inhibitory effect of n-3 PUFA and CLA, but not of n-6 PUFA such as arachidonic acid, on adhesion molecule expression can be explained, at least in part, by induction of enzymes of the cellular antioxidant system in endothelial cells, such as glutathione peroxidase and phospholipid hydroperoxyde glutathione peroxidase activities (26, 93). This induction results from stimulation of gene transcription either by the fatty acids themselves or by the conjugated dienes resulting from their peroxidation (29). The mechanism of action of these oxidation products remains to be clarified.

Despite the wealth of evidence implicating a role for dietary fatty acids in modulating endothelial function, it is still unknown whether fatty acids influence the action of insulin at the level of the endothelium. Given the recognized role of oxidant stress in mediating insulin resistance, it appears crucial to determine whether dietary fatty acids modulate insulin signaling to eNOS activation and NO production, as well as the longer term but important antiapoptotic effect of the hormone on endothelial cells.

CONCLUSION

There is growing evidence that redox signaling plays an important role in mediating pathological effects of inflammatory cytokines and other factors (hyperglycemia, fatty acids, oxidized LDL) known to be associated with cardiovascular risk. It is thought that ROS and probably RNS interfere with insulin signaling in the endothelium, leading to vascular impairment in altered metabolic states, such as obesity, diabetes, and atherosclerosis. Neutralization of these reactive species by enhancing endogenous antioxidant systems and activation of antiinflammatory pathways by insulin-sensitizing drugs both represent promising approaches to target redox imbalance in these diseased states.

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ABBREVIATIONS

AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; AP-1, activator protein-1; BH_4, tetrahydrobiopterin; CLA, linoleic acid conjugates; eNOS, endothelial nitric oxide synthase; GTPCH, guanosine triphosphate–cyclohydrolase I; H_2O_2, hydrogen peroxide; IκB, inhibitor of κB; ICAM-1, intracellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; IR, insulin receptor; IRS, insulin receptor substrate; LDL, low-density lipoprotein; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; $OONO^-$, peroxynitrite; PDK-1/2, 3′-phosphoinositide-dependent protein kinases; PI, phosphatidylinositol; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-triphosphate; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; RNS, reactive nitrogen species; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TZD, thiazolidinedione.

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Address reprint requests to:
André Marette, Ph.D.
Department of Physiology and Lipid Research Unit
Laval University Hospital Research Center
2705, Laurier Bld
Ste-Foy (Québec), Canada, G1V 4G2

E-mail: andre.marette@crchul.ulaval.ca

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