

Justification for antioxidant preconditioning (or how to protect insulin-mediated actions under oxidative stress)

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Insulin resistance is characterized by impaired glucose utilization in the peripheral tissues, accelerated muscle protein degradation, impaired antioxidant defences and extensive cell death. Apparently, both insulin and IGF-1 at physiological concentrations support cell survival by phosphatidylinositol 3 kinase-dependent and independent mechanisms. Postprandial hyperglycemia and hyperinsulinemia are found in insulin resistance, which accompanies the so-called noninsulin dependent diabetes mellitus (diabetes type 2). Evidence also indicates that increased susceptibility of muscle cells and cardiomyocytes to oxidative stress is among the harmful complications of insulin resistance and diabetes. Limited knowledge showing benefits of preconditioning with antioxidants (vitamin C, E, α -lipoic acid, *N*-acetylcysteine) in order to protect insulin action under oxidative stress prompted the author to discuss the theoretical background to this approach. It should be stressed that antioxidant preconditioning is relevant to prevention of both diabetes- and insulin resistance-associated side-effects such as low viability and cell deletion. Furthermore, antioxidant conditioning promises to provide higher efficacy for clinical applications in myoblast transfer therapy and cardiomyoplasty.

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1. Diabetes and oxidative stress

1.1 Mitochondria – the targets of diabetes and ischemia-reperfusion injury

Accelerated fatty acid (FA) metabolism as the consequence of the reduced glucose uptake during diabetes causes changes in the activity of the FA transporters, FA storage and activation of peroxisomal, mitochondrial and other enzymes involved in lipolysis and *b*-oxidation (Schmitz-Peiffer 2000). Hyperglycemia, advanced glycation end products (AGE), autooxidation of glucose, polyol

pathway (increased glucose flux through the aldose reductase pathway) and intracellular accumulation of lipids and metabolic alterations all lead to the increased formation of oxygen-derived reactive oxygen species (ROS) (Rosen *et al* 2001) which are known to abrogate the metabolic effects of insulin (Hansen *et al* 1999). Insulin deficiency and hyperglycemia lead both to activation of sympathetic system (Landsberg 2001; Bakker *et al* 2001) and renin-angiotensin system (Fukuda *et al* 2001) and endothelial dysfunction (Makimattila *et al* 1999). Diminished action of insulin, either by the absolute depletion of *b*-cells in pancreatic islets or by the insulin resistant

Keywords. Antioxidants; insulin resistance; preconditioning; ROS/RNS

Abbreviations used: AGE, Advanced glycation end products; FA, fatty acid; FKHR, forkhead-related transcription factor; IDDM, insulin-dependent diabetes mellitus; IGFs, insulin like growth factors; LA, α -lipoic acid; NO, nitric oxide; NOS, nitric oxide synthase; O_2^- , superoxide anion radical; PCD, programmed cell death; PDK1, 3'-phosphoinositide dependent kinase 1; PI-3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; RNS, reactive nitrogen species; ROS, reactive oxygen species; XO, xanthine oxidase.

state, is also potentiated by the disturbances in the regulation of intrinsic prooxidant-antioxidant homeostasis regulated by the mitochondria. Mitochondrial electron flux becomes uncoupled from ATP synthesis during hyperglycemia (Anderson 1999). Normalizing levels of mitochondrial ROS prevents glucose-induced activation of protein kinase C (PKC), formation of AGE, sorbitol accumulation and NF kappa B (NF- κ B) activation (Nishikawa *et al* 2000). Uncouplers of the mitochondrial electron chain uncoupling proteins 2 and 3 (UCP-2 and -3) seem to correct and prevent the release of free radicals from mitochondria in healthy subjects (Samec *et al* 1999) as well as stimulate glucose uptake in muscle cells (Huppertz *et al* 2001). This is not the case in type-2 diabetics (NIDDM) who suffer from the lack of induction of skeletal muscle UCP-2 and -3 expression (Vidal *et al* 1999). Therefore during diabetes the regulatory mechanisms (i.e. UCPs) fail to intervene leading to serious complications. Apparently, accelerated substrate flow from either glucose or fatty acids seem to be final cause of generation of ROS/RNS and oxidative stress.

1.2 *The links between hypoxia/hyperoxia, antioxidants and insulin action*

Intracellular hydrogen peroxide (H_2O_2) formation is a PO_2 -dependent, continuous process regulated by a non-respiratory chain heme-containing oxidase – most likely ‘low-output’ NADPH oxidase (Wenger and Gassmann 1997) but not ‘high-output’ NADPH oxidase (Wenger *et al* 1996). Other unknown and unidentified oxidases may play a similar role to NADPH oxidase (Danon 2002). The expression of carbohydrate-metabolizing enzymes in the liver is a PO_2 -dependent process with a more efficient glucagon-induced expression of the gluconeogenic rate-limiting enzyme phosphoenolpyruvate carboxykinase (PCK) at higher PO_2 . Reciprocally, insulin-induced expression of the glycolytic enzyme glucokinase was repressed by higher PO_2 (Kietzmann *et al* 1998). The role of H_2O_2 as intracellular messenger from the O_2 sensor was substantiated in the studies where H_2O_2 mimicked higher pericellular PO_2 by inhibiting insulin action, and was further corroborated by the reversal of this effect by catalase which scavenges H_2O_2 (Kietzmann *et al* 2000). Our studies on muscle cell lines have also shown inhibitory action of H_2O_2 on insulin-mediated growth promoting effects in L6 rat myoblasts/myotubes (Orzechowski and Grzelkowska 2000; Orzechowski *et al* 2002b). On the other hand, H_2O_2 was found to ameliorate differentiation of L6 muscle cells or C2C12 mouse satellite cells most likely by exerting the antimitogenic effect (inhibition of DNA synthesis) and withdrawal from cell cycle (Orzechowski *et al* 2002a). Notably, antioxidants

(sodium ascorbate, *N*-acetylcysteine) at mmol concentrations and catalase alone protected insulin activity and abolished H_2O_2 -mediated effects (Orzechowski *et al* 2002b). In addition, it appeared that antioxidants inhibited myogenesis from C2C12 satellite cells probably by elevating the number of premitotic cells and by extending mitogenesis (DNA synthesis) (Orzechowski *et al* 2001). Interestingly, insulin opposed the antimitogenic effect of H_2O_2 , which imitated higher oxygen tension (Orzechowski *et al* 2002b).

1.3 *Ischemia-reperfusion injury during insulin resistant states*

Based on experimental data and clinical reports, it has been suggested that metabolic changes occurring during the early phase of diabetes might precondition cardiac myocytes against oxidative stress (Tosaki *et al* 1996). It was postulated that the exposure to oxidative stress arising from the diabetic state (Feillet-Coudray *et al* 1999), might activate antioxidant-defence-mechanisms to balance oxidative stress (Torres *et al* 1999; Duarte *et al* 2000). However, some findings suggest increased vulnerability of diabetic subjects to the insult of oxygen toxicity (Ghalla *et al* 1998), including studies evaluating experimentally induced myocardium ischemia-reperfusion (I-R) or acute episodes of myocardial infarction (Feuvray *et al* 1979; Ko *et al* 2001). In both systems, myocytes showed functional impairment (reduced contracting force) with damage more accentuated after heart stroke under diabetic conditions (Rennert *et al* 1985). From these and other studies it seems clear that insulin deficiency during diabetes mellitus is a causal factor to developing whole-body oxidative stress (Rosen *et al* 2001) with consequent remodelling of heart structure and impaired function (Ustinova *et al* 2000; Dhalla *et al* 1998).

1.4 *Exercise, insulin sensitivity and insulin metabolic effects*

Overwhelming evidence from numerous studies shows that both an acute bout of exercise or physical training positively affects insulin action in muscle and probably other tissues (Borghouts and Keizer 2000). Thus, exercise is the most powerful agent that sensitizes tissues to insulin, probably by influencing post-receptor insulin signalling and glucose transport (Wojtaszewski *et al* 1999). Therefore it is thought that physical activity plays essential role in the treatment and prevention of insulin insensitivity. How does acute exercise and/or physical training induce such an effect? Physiological changes associated with exercise might shed light on the possible causal relationship between the increased demand for

oxygen (oxygen tension) and insulin action. In fact, even mild or moderate exercise is accompanied by oxygen debt regardless of substantial muscle hyperemia. Thus, contracting muscles should have to consume more oxygen, which is provided inadequately by circulatory, and respiratory systems. Consequently, hypoxia develops, which was described to promote insulin action at least in hepatocytes. It seems reasonable to assume that exercise-induced hypoxia in muscles also promotes insulin action or acts by itself to mimic some of the insulin-dependent effects. One very important effect of insulin is glucose transport. The observation that hypoxic conditions substantially enhance glucose uptake in muscles was reported by several authors and reviewed by Ismail-Beigi (1993). Both hypoxia and inhibitors of oxidative phosphorylation activate glucose transporters (GLUT-1 and GLUT-4) via distinct *cis*-acting sequences (Elbert *et al* 1995). The mediator of the molecular response to hypoxia is hypoxia inducing factor (HIF-1), probably the most important transcription factor that upregulates expression of GLUT-1 (Gassman and Wenger 1997). Reduced respiration with resultant decrements in ATP production in mitochondria and/or the depletion of muscle glycogen stores with exercise may lead to increased glucose transport in order to balance high demands for glucose during anaerobic metabolism (Borghouts and Keizer 2000; Diamond and Carruthers 1993). GLUT-1 mediates the basal transport of glucose (noninsulin sensitive), and together with GLUT-4 (insulin-sensitive) appears to be the primary-active-glucose transporters during early development (Hocquette and Balage 1996). Furthermore, GLUT-1 and GLUT-4 are ubiquitously expressed in almost all tissues and their genes are activated by several factors including reducing conditions (Hocquette *et al* 1996). Age, physical activity and oxidative type of muscle all were associated with increased oxygen demands and were reported to cause higher activity of glucose transporters and higher sensitivity to their ligands (Hocquette *et al* 1997; Hocquette and Abe 2000). Signal transduction pathways mediating the effect of hypoxia on GLUT-1 overexpression are, however, unknown. Whether GLUT-1 gene transcription, translation and finally translocation is triggered by reduced ATP content or rise in cytosolic Ca^{2+} or increased NADPH/NADP⁺ ratio is a matter of debate: that glucose transport was activated prior to fall in ATP as reported by Abel *et al* (2001); or was due to increased cytosolic Ca^{2+} as reported by Schaffer *et al* (2000); or was because of altered concentration of NADPH (Taha *et al* 1997). Therefore, at the moment it is not clear to what extent ATP, or redox state, or calcium current contributes to the above-described phenomenon. If the glucose uptake is upregulated during hypoxia, and the key role is played by HIF-1, it is tempting to speculate that the reducing agents that scavenge H_2O_2 (mimic

of O_2 abundance) would activate glucose transport. In fact, α -lipoic acid (LA, thioctic acid) an effective metabolic antioxidant in its reduced form (dihydrolipoic acid) and modulator of cellular redox state, was shown to stimulate skeletal muscle glucose uptake (Estrada *et al* 1996). Although LA stimulated GLUT-4 translocation and activation at least in part via an insulin signalling pathway (Konrad *et al* 2001), the effect was thought to be noninsulin-dependent since it occurred in insulin-resistant state (Khanna *et al* 1999). Surprisingly, growing evidence also points to the involvement of ROS/RNS as potent activators of glucose uptake, but this type of stimulation is mediated by noninsulin-dependent facilitative-glucose transport in several cell types, including muscles (Bedard *et al* 1997; Kozlovsky *et al* 1997; Blair *et al* 1999; Prasad and Ismail-Beigi 1999). The results of studies with ras-transformed fibroblasts suggest the involvement of MAP signalling in increased glucose transport (Onetti *et al* 1997). Since exercise as well as antioxidants causes substantial activation of the MAP cascade, this molecular mechanism of increased glucose transport is proposed in contracting skeletal muscle (Somwar *et al* 2000; Balon and Yerneni 2001).

Hypoxia also activates GLUT-1 and GLUT-4, suggesting a role for inhibitors/uncouplers of oxidative phosphorylation (Shetty *et al* 1992). In contrast to GLUT-1, hypoxia-induced glucose uptake by GLUT-4 is distinct from insulin-mediated. It remains to be determined whether the hypoxia-associated exercise allows insulin to act more strongly on muscles because the reducing conditions favour release of glucose transporters and/or increase their expression. Regular or even short-term exercise augments insulin-stimulated PI-3K activity (Houmar *et al* 1999; Kirwan *et al* 2000), whereas type 2 diabetes does not (Kim *et al* 1999). PI-3K is the essential kinase in the insulin-mediated signal transduction cascade in skeletal muscle (Wick and Liu 2001). Alternatively or additionally, muscles subjected to mechanical activity release growth factor(s) that mimic insulin effect. Stretched muscles express mRNA derived from IGF-I gene called mechano growth factor (MGF), which might explain hypertrophy of exercised muscle but not all muscles of the limb (Goldspink 2002). IGFs are a group of cytokines that show mimicry to insulin, cross-react with insulin receptor and vice versa, and utilize the insulin-dependent signalling pathway (Schlessinger 2000). Another important issue relevant to endurance training in muscle is also increased expression of mitochondrial enzymes (Chandwaney *et al* 1998) that could be induced by hypoxia/anoxia. It makes physiological sense that a compensatory mechanism opposing compromised oxygen utilization during hypoxia/anoxia leads to switching on the genes of the mitochondrial enzymes, although the exact nature of this regulation is unknown. It has long

been appreciated that thyroid hormones act through transcription factor, Tfam, to control the expression of respiratory enzymes in muscle mitochondria (Hood 2001). This phenomenon, however, occurs in muscles that are not subjected to stretch (Wrutniak-Cabello *et al* 2001). On the other hand, it has recently been reported that insulin activates the synthesis of muscle mitochondrial enzymes but not of other protein fractions in skeletal muscle (Boirie *et al* 2001).

In conclusion, either it is physical exercise, or it is insulin that are the major regulatory factors for mitochondrial protein synthesis in skeletal muscle. Muscle-specific action of insulin indicates the considerable role of exercise as surrogate/or complementary to insulin. It remains to be ascertained how antioxidants, which were reported to increase insulin sensitivity (Rosen *et al* 2001), act through a molecular mechanism similar to the one termed 'athletic paradox'. The latter stands for elevated antioxidant defences in skeletal and heart muscle after acute or long-term exercise, which is believed to be a consequence of ROS/RNS priming and modulation of antioxidant gene expression (Chandwaney *et al* 1998; Wilson and Johnson 2000).

1.5 Diabetes and the molecular mechanism of insulin action, the role of ROS

Apoptosis or programmed cell death (PCD) is a well recognized process of cell deletion, induced either by intrinsic physiological signals or by cytopathic/cytotoxic agents. The hallmark of apoptosis is a mitochondrial permeability transition (MPT), Ca^{2+} -mediated loss of mitochondrial membrane selectivity and integrity that allows ROS leakage throughout the pores into cytoplasm (Lemasters *et al* 1998). Developing intracellular oxidative stress (Simon *et al* 2000) and the release of cytochrome *c* (Cyt C), apoptosis activating factor (APAF-1), apoptosis inducing factor (AIF), and procaspase-3 allows the formation of the apoptosome (a complex of APAF-1-Cyt C-dATP) which initiates the final step of apoptosis – i.e. the activation of caspase-9 (one among several cysteine aspartases) which in turn proteolytically activates caspase-3. Similarly caspase-3 by proteolytic cleavage activates several substrates (enzymes) of the execution phase of PCD, which result in cell blebbing and oligonucleosomal DNA fragmentation (Oberholzer *et al* 2001). It is hypothesized that the crucial and irreversible step in the process of apoptosis is the formation of pores in the mitochondrial membrane and this almost entirely depends on: (i) the activity of pro-apoptotic protein Bad, (ii) the intracellular translocations of pro-apoptotic protein Bax, and (iii) their interaction with anti-apoptotic proteins Bcl-2 and Bcl-X_L (Motyl 1999). Other pro-

apoptotic members of Bcl-2 family proteins (Bid, Bik, Bak) are also of considerable importance in megapore formation. In general, homodimerization of Bax or other pro-apoptotic proteins at different subcellular localizations, particularly at mitochondria, promotes apoptosis whereas heterodimerization with Bcl-2 or Bcl-X_L prevents the process of PCD (Hockenbery *et al* 1993). In nonphosphorylated form Bad proteins are active and sequester Bcl-2 or Bcl-X_L so apoptosis initiated by specific stimuli might proceed, otherwise, when Bad is phosphorylated Bcl-2 or Bcl-X_L proteins which are not assembled with Bad translocate to form heterodimers with Bax and the process of PCD is blocked (Hsu *et al* 1997). One of the most powerful and intrinsic inhibitors of Bad protein is protein kinase B (PKB), also termed Akt. This threonine-serine kinase phosphorylates Bad at serine 136 (Ser¹³⁶) residue and creates binding site for a 14-3-3 adaptor protein (Datta *et al* 1997), which in turn retains Bad protein in the cytosol and protects against dimerization with either Bcl-2 or Bcl-X_L. The same kinase also phosphorylates forkhead-related transcription factor (FKHR), which is therefore immobilized in the cytoplasm by binding to the 14-3-3 protein (Brunet *et al* 1999). In the aforementioned circumstances FKHR, is no more active and does not migrate to nucleus to transactivate pro-apoptotic genes (Kops *et al* 1999). The last and probably least known action of PKB is direct targeting of caspase-9, which by phosphorylation remains in inactive form (Cardone *et al* 1999). PKB is the crucial target in the signalling pathway of insulin and several other cytokines, including insulin like growth factors (IGFs) recognized as one of the key mediators of the phosphoinositide 3-kinase (PI-3K) signal (Somwar *et al* 1998; Rommel 2001). Initially, the PI-3K mediates the transfer of phosphate groups to phosphatidylinositol residues located in the plasma membrane (3'-OH position of the inositol ring), then by plextrin homology (PH) domain PKB enables assembly with 3'-phosphoinositides generated, and 3'-phosphoinositide dependent kinase 1 (PDK1) phosphorylates PKB at threonine 308 (Thr³⁰⁸) residue, and finally PKB is activated by autophosphorylation or phosphorylation by hypothetical PDK2 at serine 473 residue (Ser⁴⁷³) (Roymans and Slegers 2001; Wick and Liu 2001).

2. Insulin in action

2.1 Antiapoptotic factor and potent inducer of antioxidant defences

Growing evidence suggest the importance of insulin and IGFs in the inhibition of apoptosis in different cell types, including muscle cells and cardiomyocytes (Chakra-

varthy *et al* 2000; Lawlor and Rotwein 2000a). Furthermore, it has been shown that insulin or IGFs prevent myoblasts, satellite cells and cardiomyocytes from oxidative stress-induced apoptosis through activation of PI-3K/PKB (Aikawa *et al* 2000; Lawlor and Rotwein 2000b; Jonassen *et al* 2001). Lack of insulin or IGFs as well as addition of inhibitors of PI-3K or mammalian target of rapamycin (mTOR) leads to extensive cell death, whereas pretreatment of cells with IGF-1 suppresses H₂O₂-induced apoptosis by subsequent inhibition of Bax expression (Napier *et al* 1999; Hong *et al* 2001). Additionally, it appears that IGF-1 induces a cAMP-response element (CRE) site-containing Bcl-2 promoter, and the regulation of Bcl-2 expression by IGF-1 involves a signalling cascade mediated by PI-3K/PDK1/Akt/CREB (cAMP-response element-binding protein) (Pugazhenthii *et al* 2000). Thus, insulin and/or IGFs improve the intracellular antioxidant status by playing the pivotal role in the PKB-mediated expression of Bcl-2 protein that protects from the intrinsic oxidant pathway originating in mitochondria. Bcl-2 protein prevents the escape of ROS by opposing the oxidative-stress-induced pro-apoptotic action of Bax (Hong *et al* 2001). This phenomenon is not unique to striated muscle cells because Akt-dependent anti-apoptotic action was observed in other cell types (Aikin *et al* 2000; Park *et al* 2000). At present, it is generally believed that the insulin and IGF-1 anti-apoptotic signalling is initiated by the interaction of their cognate receptors to phosphorylate IRS proteins at sites that facilitate interaction with the PI-3K and, subsequently, PKB/Akt. However, factors with the ability to activate the small GTPase Rac seem to activate other alternative pathways to inhibit apoptosis (Boehm *et al* 1999). This latter possibility is associated, however, with the intracellular generation of ROS by NADPH oxidase (Berk 1999), activation of p38 MAP kinase (Zhang *et al* 2001a) and finally anti-apoptotic transcription factor NF- κ B (Ho *et al* 1999). Activated NF- κ B translocates to the nucleus and transactivates several genes of antioxidant defence. Not surprisingly, then, both antioxidants and oxidants were found to induce similar protective effects as insulin and other cytokines against oxidative insult.

2.2 The role of prooxidant-antioxidant homeostasis in cell survival

The powerful antioxidant, α -lipoic acid, prevented H₂O₂-induced cell death of cortical neurons (Zhang *et al* 2001a). Similarly, but, probably through other mechanisms, oxidized low-density lipoprotein prevented macrophage apoptosis through PKB activation (Hundal *et al* 2001). Thus, numerous reports describing benefits of preconditioning with mild oxidative stress (Okubo *et al* 1999)

only seemingly contradict the results of studies with antioxidant preconditioning. Ischemia-reperfusion injury is almost entirely caused by free radicals. Different approaches to limit generation of oxidative stress would be equally important. Hypoxia, which was indicated to promote insulin action, is able to promote oxidative stress at the same time by the activation of xanthine oxidase (XO), which oxidizes purines and reduces oxygen (O₂) to form superoxide anion radical (O₂⁻) (Hoshikawa *et al* 2001). Antioxidants (NAC) or XO inhibitors (allopurinol) efficiently antagonize hypoxia-associated oxidative stress *in vivo* (Shadid *et al* 1999a). Inside injured muscles, oxidized myoglobin generates Fe³⁺-mediated ROS which contribute substantially to the oxidant stress (Gunther *et al* 1999). Iron-chelator deferoxamine (DFO) was shown to exert protective effect on myocardial performance after post-ischemic reperfusion injury (Shadid *et al* 1999b). Iron-complexed neurotransmitters, such as dopamine: iron(III) complex, also showed high superoxide scavenging effectiveness to protect isolated hepatocytes against hypoxia-reoxygenation injury (Siraki *et al* 2000). Antioxidants, such as L-carnithine or vitamin C, were reported to be either antiapoptotic or able to correct insulin insensitivity in endothelium of coronary vessels, respectively (Andrieu-Abadie *et al* 1999; Hirashima *et al* 2000). Importantly, it was demonstrated that insulin actually suppresses generation of O₂⁻ in diabetic rat glomeruli (Chen *et al* 2001), which is in accordance with the previously-mentioned suggestion that insulin promotes survival of cells by elevating their antioxidant capacity.

2.3 Hyperglycemia and insulin action

Hyperglycemia has often been reported to induce oxidative stress by several different routes of action, but detailed description of this process is beyond the scope of this paper. The data demonstrate that hyperglycemia in muscles might inhibit insulin-dependent signalling that activates PKB. Subsequently, almost all insulin-mediated physiological effects (except antilipolytic) are diminished including the anti-apoptotic action of insulin (Oku *et al* 2001). PKB inhibition was shown to abrogate insulin-mediated stimulation of glucose transport and GLUT4 membrane translocation (Smith *et al* 2000). Furthermore in sucrose-fed rats, hyperglycemia-induced functional and structural modifications in cardiac muscle were associated with insulin resistance (Dutta *et al* 2001). Some of the pathological consequences of insulin resistance seem to be related to the lack of the inhibition of glycogen synthase kinase-3 (GSK-3) during insulin-resistant states. When this kinase is not suppressed by insulin, it mirrors the situation of oxygen abundance and moderate oxidative stress which in turn promote activity of glucose-6-

phosphatase and PEPCK – the step limiting enzymes of *de novo* glucose formation (Lochhead *et al* 2001). Apparently, either type 2 diabetes or insulin resistance both lead to enhanced gluconeogenesis, i.e. elevated glucose production and release, therefore they exaggerate the existing hyperglycemia. Three of the pathways of hyperglycemic damage; namely, PKC activation, increased formation of glucose-derived advanced glycation end products, and increased flux through the aldose reductase pathway, were efficiently blocked by normalizing mitochondrial superoxide production (Nishikawa *et al* 2000). The latter observation is a milestone in recognizing the important role of oxidative stress in the retarded insulin-stimulated metabolic regulation.

2.4 Hyperinsulinemia – how excess of insulin affects basic physiological functions

One could bear in mind that excess insulin should make cells less susceptible to oxidative stress and resultant disorders. Accumulating evidence suggests, however, that high insulin level can additionally predispose or precede several systemic disorders, especially those related to impaired systemic circulation. Hypertension and increased risk of stroke were found in diabetic and hyperinsulinemic nondiabetic subjects to be associated with an elevated level of proinsulin (Lindahl *et al* 2000). Hypertension is most likely caused by hyperglycemia, since exogenous hyperinsulinemia alone does not elevate blood pressure. It is assumed therefore, that insulin is a potent activator of nitric oxide (NO[•]) synthase (NOS) activity, and NO[•] causes vasodilatation (Ignarro *et al* 1987). If however NOS is inhibited, new conditions appear that unmask the hypertensive effect of chronic hyperinsulinemia (Bursztyjn *et al* 2000). Whether increased NO[•] generation, induced by hyperinsulinemia, enables development of oxidative stress is unclear, however, only minute amounts of NO[•] are formed by constitutive NOS (eNOS, nNOS) in comparison to the burst of NO[•] released by inducible NOS (iNOS). Theoretically, abundance of NO[•] may bypass the benefits of antioxidative action of insulin, since NO[•] is a free radical that inhibits respiration and by rapid reaction with superoxide might form peroxynitrite (ONOO⁻) (Ishii *et al* 1999). Both NO[•] and ONOO⁻ by nitration reactions inactivate several important antioxidant enzymes, such as peroxisomal catalase (Brown 1995) and mitochondrial superoxide dismutase (MnSOD) (MacMillan-Crow *et al* 1996) respectively. Another possibility is that hyperinsulinemia-associated oxidative stress observed during insulin resistant states is caused by repressive action of RNS on ATP formation and elevated O₂^{-•} formation in mitochondria. Additionally, high insulin has been reported to

upregulate desaturation reactions which together with accelerated mitochondrial peroxidation allow extensive peroxidation of lipoproteins and lipid peroxides which is associated with the ethiology of atherosclerosis (Facchini *et al* 2000) and ageing (Wick and Liu 2001). In fact in lower eukaryotes, a reduction in signalling through IGF-1 receptor or decreased insulin signalling results in promoting an extended life span of *Cenorhabditis elegans* and *Drosophila*, respectively (Wick and Liu 2001). Aforementioned observations offer an attractive explanation for the antiageing effect of calorie restriction (Lee *et al* 1999). Diet restriction was found to alter glucose use by increased muscle insulin sensitivity mediated by overexpression of GLUT4 transporter protein (Dean *et al* 1998). In contrast, acute hyperinsulinemia failed to upregulate GLUT4 protein expression in skeletal muscle or adipose tissue (Balage *et al* 1998). In conclusion, insulin deprivation during diabetes mellitus and insulin resistance-associated hyperinsulinemia apparently both lead to oxidative stress, which is exacerbated by accompanying hyperglycemia. A balance in carbohydrate-stimulated release of insulin and insulin clearance from circulation assure proper control of antioxidant capacity in tissues. Hypothetically, harmful consequences of oxidative stress could be ameliorated by repair of antioxidant defence mechanisms. Repair strategy might include overexpression of copper transport and antioxidant proteins such as metallothioneins (Wang *et al* 2001), priming with oxidants such as lipopolysaccharide (LPS) (Ahmad *et al* 2000), conditioning hypoxia (Rutrich and Krug 1999; Sugawara *et al* 2000), or simply be preconditioning with the moderate and high doses of vitamin C and/or vitamin E or other antioxidants (Hirashima *et al* 2000; Title *et al* 2000; Rossig *et al* 2001; Bordoni *et al* 2002).

3. The role of antioxidants

3.1 Antioxidant defences during diabetes

The oxy-radical loaded heart and other organs may become primed to activate antioxidant systems (Torres *et al* 1999). Diabetic hearts were less susceptible to oxidative stress where antioxidant defences had been exaggerated. Higher levels of reduced glutathione (GSH)/ascorbic acid and α -tocopherol were found in diabetic hearts, whereas insulin treatment elevated myocardial vitamin C and decreased vitamin E (Ko *et al* 2001). In experimentally induced diabetic rats, catalase activity in blood was elevated (Torres *et al* 1999) but not glutathione peroxidase (GPx) or superoxide dismutase (SOD) (Kesavulu *et al* 2000; Doi *et al* 2001). In contrast, heart insufficiency developed when antioxidant capacity was compromised in chronic diabetes (Opara *et al* 1999; Doi *et al* 2001).

Thus under the diabetic state antioxidants play a crucial role in the protection of the heart muscle cells against oxygen-derived radicals. Acute effects of oxidative stress differ from longterm exposure to reactive oxygen/nitrogen species (ROS/RNS). Cells may benefit from short-term preconditioning with moderate levels of ROS/RNS, whereas prolonged exposure might be devastating.

We admit, therefore, that tissue antioxidant defences respond to oxidative stress by gradually increased the capacity although if the latter is overcome by the oxygen insult oxidative stress of higher magnitude develops, and injuries caused by ROS/RNS become substantial and irreversible. Are there any simple, straight solutions available to cope with the issue of oxidative stress-threatening muscle cells? Accumulating evidence indicates promising perspectives from the studies with antioxidants used to protect them.

3.2 Preconditioning with antioxidants – an alternative approach against diabetes?

Dietary or pharmacological treatments of animals with antioxidants attenuated the injuries caused by oxidative stress under diabetes. The development of insulin-dependent diabetes mellitus (IDDM) is believed to be the outcome of **b**-cell deletion in pancreatic islets by an autoimmune response that triggers an exaggerated inflammatory reaction mediated by NF- κ B and accompanied by the release of excess ROS/RNS that are cytotoxic to **b**-cells (Ho and Bray 1999). Experimental IDDM caused by the application of alloxan or streptozotocin also results in hydroxyl radical- (\cdot OH) or NO \cdot -abundance and **b**-cell death. Antioxidants efficiently scavenge free radicals and preserve **b**-cells. In particular melatonin used in the pre-treatment (preconditioning) was reported to act as a shield to prevent **b**-cells elimination (Andersson and Sandler 2001; Ebel *et al* 2000). Preconditioning with antioxidants, such as *N*-acetylcysteine, vitamin C and/or vitamin E suppressed apoptosis of **b**-cells in transgenic mice to whom non insulin dependent diabetes mellitus (NIDDM) developed as the result of hyperglycemia toxicity. Thus growing evidence suggests the role of oxidative stress in **b**-cell dysfunction, and points to the usefulness of antioxidant preconditioning in order to preserve *in vivo* **b**-cell function (Kaneto *et al* 1999).

4. Perspectives

Cautious investigation of dose-dependent insulin-induced biological effects allows to hypothesize that both deficiency of blood serum insulin (hypoinsulinemia) and excess of insulin (hyperinsulinemia) leads to similar harmful consequences that are associated with the oxidative in-

sult. Furthermore, the imbalance of redox homeostasis resulted in oxidative stress mimics the situation where insulin level is either below or above the physiological level. During hypoinsulinemia, insulin antioxidative effect transmitted by PKB to protect cells from oxidative insult of mitochondrial origin is reduced, whereas hyperinsulinemia overrules antioxidative effect of insulin by pro-oxidative action resulted from insulin-dependent overexpression of respiratory chain enzymes in mitochondria. In other words, the side effect of hyperinsulinemia is the accelerated formation of ROS in mitochondria which is not counterbalanced by the PKB-dependent antioxidative mechanism. It remains to be established why such a vital hormone, as insulin, can cause rather opposite consequences to intracellular redox homeostasis that depend on insulin concentration. Based on personal observations and literature data of others, it is suggested that sensitivity to insulin, modulated by ROS, might be crucial to diversification of insulin-dependent effects. Therefore, antioxidants preconditioning might be a straightforward and efficient route to prevent insulin action in cells, allowing them to play their physiological functions, especially useful to improve the achievements of cell transfer therapy (Skuk and Tremblay 2000; Zhang *et al* 2001b).

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