EBM

Experimental Biology and Medicine

Mitochondrial Nitric Oxide Synthase: A Masterpiece of Metabolic Adaptation, Cell Growth, Transformation, and Death

Paola V. Finocchietto, Maria C. Franco, Silvia Holod, Analia S. Gonzalez, Daniela P. Converso, Valeria G. Antico Arciuch, Maria P. Serra, Juan J. Poderoso and Maria C. Carreras

Experimental Biology and Medicine 2009, 234:1020-1028.
doi: 10.3181/0902-MR-81

MINIREVIEW

Mitochondrial Nitric Oxide Synthase: A Masterpiece of Metabolic Adaptation, Cell Growth, Transformation, and Death

Paola V. Finocchietto,*,[1] Maria C. Franco,* Silvia Holod,*,† Analia S. Gonzalez,*,† Daniela P. Converso,* Valeria G. Antico Arciuch,* Maria P. Serra,* Juan J. Poderoso,*,‡ and Maria C. Carreras*,†

Laboratory of Oxygen Metabolism, University Hospital, †Department of Clinical Biochemistry, School of Pharmacy and Biochemistry, and ‡Department of Internal Medicine, School of Medicine, University of Buenos Aires, CONICET, 1120 Buenos Aires, Argentina

Mitochondria are specialized organelles that control energy metabolism and also activate a multiplicity of pathways that modulate cell proliferation and mitochondrial biogenesis or, conversely, promote cell arrest and programmed cell death by a limited number of oxidative or nitrative reactions. Nitric oxide (NO) regulates oxygen uptake by reversible inhibition of cytochrome oxidase and the production of superoxide anion from the mitochondrial electron transfer chain. In this sense, NO produced by mtNOS will set the oxygen uptake level and contribute to oxidation-reduction reaction (redox)–dependent cell signaling. Modulation of translocation and activation of neuronal nitric oxide synthase (mtNOS activity) under different physiologic or pathologic conditions represents an adaptive response properly modulated to adjust mitochondria to different cell challenges. Exp Biol Med 234:1020–1028, 2009

Key words: mtNOS; nitric oxide; peroxynitrite; mitochondria; insulin; thyroid

We acknowledge financial support from University of Buenos Aires (UBACyT M063 and M058), Agencia Nacional para la Promoción Científica y Tecnológica, PICT 8468 and 14199, CONICET (PIP 5495), and Fundación Pérez Companc, Buenos Aires, Argentina.

[1] To whom correspondence should be addressed at Laboratory of Oxygen Metabolism, University Hospital, Cordoba 2351, 1120 Buenos Aires, Argentina. E-mail: pfinocchietto@hotmail.com; carreras@ffyb.uba.ar

DOI: 10.3181/0902-MR-81
1535-3702/09/2349-1020$15.00

Mitochondrial Nitric Oxide Synthase

Mitochondria are the central organelles in cell bioenergetics. During the past 10 years, significant regulatory effects of nitric oxide (NO) on mitochondrial respiration became evident as resulting from its high-affinity binding to cytochrome oxidase, the final electron acceptor of the mitochondrial electron transfer chain (1–3). Synthesis of NO from L-arginine and O_2 is catalyzed by nitric oxide synthases (NOS) (4). There exist three canonical isoforms: neuronal (nNOS) or type I, inducible (iNOS) or type II, and endothelial (eNOS) or type III, and a significant number of spliced and post-translationally modified variants.

The first reports of the presence of an NOS isoform in mitochondria were from Bates *et al.*, who demonstrated in 1995 the immunocytochemical localization of nitric oxide synthase in nonsynaptosomal rat brain and liver mitochondria (5). After that, several groups showed the functionality of the enzyme present in rat liver mitochondria, its Ca^{2+}-dependency, its localization at the inner mitochondrial membrane, and that mtNOS continuously controls mitochondrial respiration (6–9).

Because NOS isoforms are encoded by nuclear and not by mitochondrial DNA, the mitochondrial variant emerged as one of the canonical NOS synthesized in the cytosol and afterward targeted to mitochondria.

Considering the complexity of the translocation process and the submitochondrial localization of the translocated protein in the different tissues, some controversies arose in the past about the identity of the mitochondrial NOS. In this way, some groups reported early the immunocytochemical

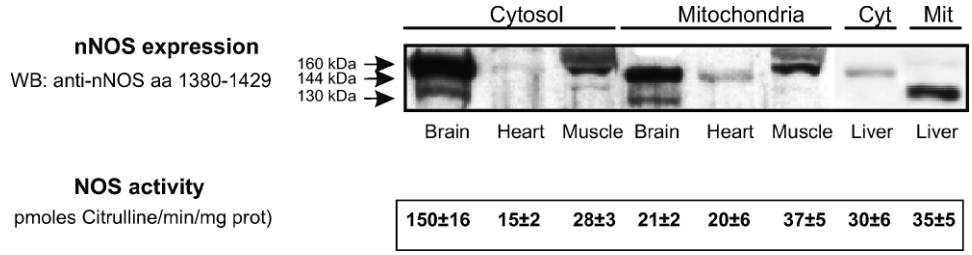

Figure 1. mtNOS expression and activity in different rat tissues. Representative Western blot of cytosolic and mitochondrial proteins separated by SDS/PAGE immunologically detected with an antibody directed to 1380–1429 C-terminal domain of nNOS α. Ca^{2+}-dependent NOS activity was determined by [^{3}H]-L-citrulline assay.

localization of eNOS in the inner mitochondrial membrane of rat brain, kidney, liver, and skeletal muscle (10, 11), while others found nNOS (12–14) or iNOS (15) by immunohistochemistry or Western blotting. Nevertheless, recent evidence confirmed nNOS as the protein completely incorporated to the inner membrane in a physiologic context. In an important contribution, Giulivi and coworkers (16) sequenced rat liver mtNOS and identified the enzyme as an inner membrane integral protein and as the transcript of nNOS, splice variant α, myristoylated and phosphorylated. Decisive evidence was provided by Kanai *et al.* (2001) with the electrochemical determination of the Ca^{2+}-induced NO release from a single mouse heart mitochondrion, a process that was abolished in nNOS –/– knockout (KO) mice (17). The nNOS KO mouse model utilized by the authors consisted of deletion of exon 2 of the nNOS gene. In this model, the nNOS α variant is not expressed, while the other nNOS variants β and γ are present. Thus, the results indicated that mtNOS in mouse heart was the product encoded by the nNOS α gene. In this way, our current studies emphasize the importance of the processing of PDZ domain in nNOS translocation to mitochondria (18). This processing should explain the differences observed between cytosol and mitochondrial nNOS, after its translocation to the inner mitochondrial membrane. In agreement, Riobó *et al.* (2002) described an nNOS variant in rat brain with an apparent molecular weight of 144 kDa, mainly located in the inner mitochondrial membrane that is distinct from 157 kDa cytosolic nNOS (12). In this context, Carreras *et al.* showed nNOS expression in rat gastrocnemius muscle mitochondria with an apparent molecular weight of 159 kDa (14); recently, Finocchietto *et al.* (2008) confirmed the decrease of mtNOS expression and activity by direct electroporation of nNOS siRNA in rat gastrocnemius muscle (19). Thus, most evidence supports the notion that mtNOS is mostly a variant of nNOS α that could be subjected to post-translational modifications that lead to different apparent molecular weights in the different cell types and organs (Fig. 1).

In pathologic conditions, such as endotoxemia, we and others have shown that iNOS can also be present in mitochondria and contribute to mitochondrial damage observed in experimental sepsis models (20, 21).

The mechanism of nNOS internalization into mitochondria is still unknown. We have recently observed that mtNOS is not detected in rat liver and brain mitochondria by anti-nNOS antibodies against the N-terminal region (aa 1–100, corresponding to the PDZ domain), while it is detected by antibodies against internal and C-terminal domains (12, 18). These data suggest that the processing of cytosolic nNOS isoform during the import to rat liver and brain mitochondria requires the loss of the PDZ domain that could account for the reduction of their apparent molecular weight. Post-translational modifications like Akt-dependent phosphorylation of Ser 1412 or acylation could regulate nNOS import into mitochondria. Since nNOS has no N-terminal presequence for mitochondrial import, it is likely that internal hydrophobic motifs determine the traffic to the organelles and that it may be related to the interaction with cytosolic proteins like caveolin-1 and distrophyn, as well as chaperones like Hsp90 and Hsp70. In this sense, Hsp90 is tightly bound to nNOS, protects the enzyme from calpain degradation (22), and could favor the interaction with Akt as it has previously been reported for eNOS (23). In several rat models (such as hypothyroidism, hyperthyroidism, and liver development), Hsp90 expression is inversely related to nNOS translocation into mitochondria (24). Similarly, intact cardiomyocytes isolated from dystrophin-deficient (*mdx*) mice are also deficient in cellular endothelial NOS, but overexpress mtNOS (17).

However, some authors were not able to detect expression or activity of mtNOS in rat or mice tissues (25). This controversy may result from the precise regulation of translocated nNOS content in different adaptive conditions; mtNOS is low in embryos and increases during postnatal development, hypothyroidism, and aging, or at environmental changes like cold exposure or low O_2 tension, and it is also modulated by several hormones (8, 12, 15, 19, 26, 27).

Recently, it has been proposed that a mammalian ortholog of *AtNOS*1 (a plant NOS gene) is one of candidates of mtNOS. The mouse ortholog of *AtNOS*1 (mAtNOS1) is localized in the inner mitochondrial membrane in fibroblast cells (COS1) and several organs of embryonic mouse, including the brain. However, the functions of mAtNOS1 for mammalian cells remain largely unknown (28).

The importance of mtNOS with respect to the other sources of mitochondrial NO is that:

1. mtNOS operates at physiologic O_2 tension;

2. mtNOS modulates mitochondrial oxygen utilization by NO flow directly driven to the electron transfer chain;

3. enzyme content is transcriptionally modulated by nNOS gene or by post-translational changes;

4. depending on mitochondrial Ca^{2+} content, mtNOS is constitutively active, differing from other enzymatic or nonenzymatic sources that provide a burst of NO;

5. clearance of mitochondrial NO is regulated by an increase of mitochondrial superoxide anion stimulated by NO itself;

6. mitochondrial NO signaling and NO-derived production of oxygen species could depend on a continuous increase of NO yield rather than on the NO boosted in endothelium by autacoids or by stimulation of synaptic NMDA receptors (29).

mtNOS and the Regulation of Oxygen Uptake

The mitochondrial respiratory chain is essential to obtain energy from nutrients in the form of NADH and $FADH_2$, reducing O_2 to H_2O and generating a membrane potential across the inner mitochondrial membrane, which drives the formation of ATP from ADP. ADP, O_2, and more recently NO, are considered to be the main regulators of this process (30). NO reversible binds to Cu^{2+}-B center of cytochrome oxidase and inhibits electron transfer to O_2 and respiration in rat skeletal muscle, liver, and heart mitochondria (31–35). Similar effects were described in the perfused rat heart (36). NO-dependent inhibition of O_2 uptake is achieved at low and physiologic NO concentrations (20–40 n*M*); 50–100 n*M* NO inhibits by half cytochrome oxidase activity. In addition, NO exerts its effects on other mitochondrial components as well; at 0.3–0.5 μmol, NO inhibits electron transfer between cytochromes *b* and c_1; moreover, as we reported for the first time, exposure of rat liver or skeletal muscle mitochondria to NO markedly increases the production rate of O_2^- and H_2O_2 (36). At 20–50 n*M* NO concentrations, most mitochondrial NO is utilized in the formation of H_2O_2 (33), relying on the inhibition of cytochrome oxidase and complex III. At higher NO concentrations, NO and derived-O_2^- react, and NO utilization is mainly driven to $ONOO^-$ formation, a powerful oxidant and nitrating agent (37). Thus, high mtNOS content conducts to NO excess and to high harmful $ONOO^-$ oxidant, and inhibits NADH dehydrogenase activity at mitochondrial complex I by nitrosylation or nitration, leading to reduced oxygen uptake, as it was reported in hypothyroidism (24, 38), and it is a hallmark of both experimental and clinical neurodegenerative diseases like Parkinson disease (39).

The regulatory properties of mtNOS on O_2 uptake have been explored by our group in different experimental models. In hypothyroid rats, we reported an increased expression and activity of liver mtNOS that was completely reverted by hormone replacement (24). The activity of mtNOS correlated with both decreased systemic and mitochondrial O_2 uptake that almost returned to control values when the animals were treated with N^{ω}-nitro.L-arginine (L-NAME) without affecting the thyroid status, suggesting a relevant role of NO in the hypothyroid phenotype (Fig. 2). In contrast, rats treated with thyroid hormone presented lower mtNOS activity and higher mitochondrial oxygen uptake. Similar changes were observed by our group in rats acclimated to a cold environment; after a week of exposure to 4°C, liver and muscle mtNOS increased, allowing high energy intake, which favored the synthesis of fat insulation, to maintain the body temperature (27).

Furthermore, Valdez *et al.* reported that heart mtNOS expression was selectively regulated by O_2 availability in hypobaric conditions and the activity was 20–60% higher in hypoxic rats than in control animals, depending on age (15).

mtNOS and the Mitochondrial Generation of Active Oxygen Species

In 1972, Boveris *et al.* reported that mitochondria produce oxygen-active species by auto-oxidation of intermediary ubisemiquinone, a transitional oxidation-reduction reaction (redox) state of membrane ubiquinol (40). About 2–3% of utilized O_2 undergoes reduction to one electron by ubisemiquinone forming O_2^-. The mechanistic basis for the NO-induced formation of intramitochondrial O_2^- and $ONOO^-$ includes three cooperative actions: (i) NO inhibits cytochrome oxidase and increases the reduction levels of the components of the electron transfer chain, including ubiquinol and ubisemiquinone, on the substrate side of cytochrome oxidase; and (ii) NO inhibits the electron transfer chain in complex III at the cytochrome *b*-c_1 region (2, i), increasing the levels of ubisemiquinone which, on auto-oxidation, reduces O_2 to O_2^-; and (c) NO reacts directly with ubiquinol to produce nitroxyl anion and ubisemiquinone (33, 36, 40, 41).

$$UQ^{\cdot -} + O_2 \rightarrow UQ + O_2{}^-$$

(reaction 1)

$$UQH + NO \rightarrow NO^- + UQH^{\cdot -}$$

(reaction 2)

$$O_2{}^- + NO \rightarrow ONOO^-$$

(reaction 3)

$$O_2{}^- + O_2{}^- + 2H^+ \xrightarrow{Mn-SOD} H_2O_2 + O_2$$

(reaction 4)

Considering the rate constants of reaction 3 and 4 ($2 \times 10^{10}\ M^{-1}\ sec^{-1}$ and $2.3 \times 10^9\ M^{-1}\ sec^{-1}$), O_2^- participation in mitochondrial metabolism will depend on NO and Mn-

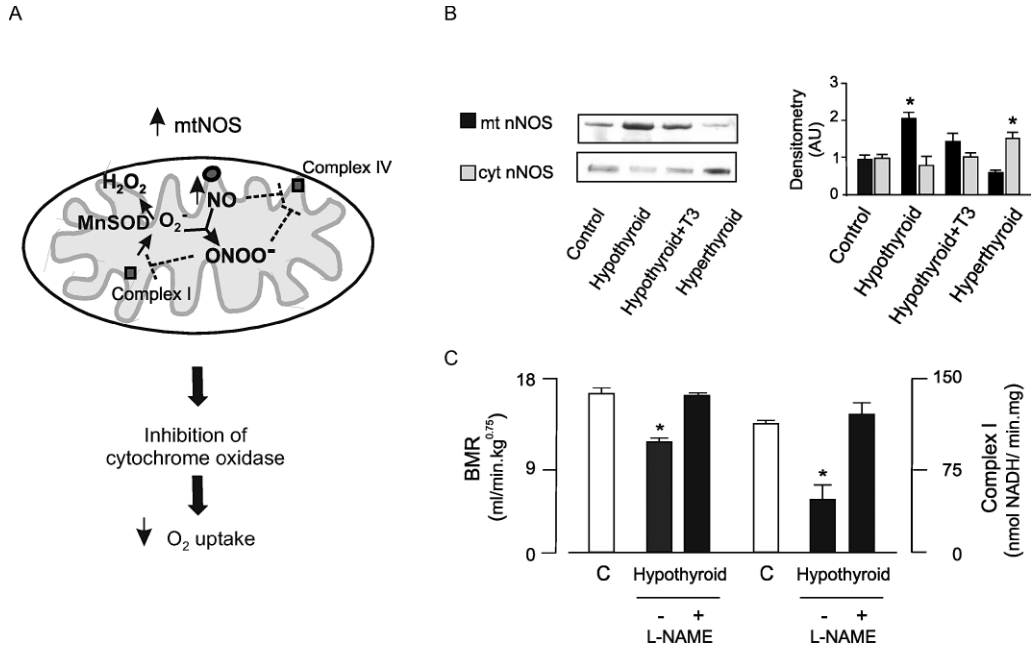

Figure 2. mtNOS and the regulation of oxygen uptake. (A) Scheme of NO effects on mitochondrial oxygen uptake and reactive species formation. (B) Representative Western blots of cytosolic and mitochondrial nNOS at different thyroid status. (C) Effect of the nNOS inhibitor N^{ω}-nitro-L-arginine (L-NAME) in basal metabolic rate and complex I activity of hypothyroid rats. Hypothyroid group represents rats treated with 0.02% methimazole (w/v) in drinking water for 28 days and hypothyroid + L-NAME group represents rats treated with 0.75 mg/ml L-NAME in drinking water in the last 21 days of methimazole treatment. Data are mean ± SEM. * denotes $P < 0.05$ after one-way analysis of variance and *post hoc* Dunnett test. This figure was reproduced in part from the article by Franco *et al.* in the *Journal of Biological Chemistry* 281:4779–4786 (24), with permission of the journal.

SOD concentrations, leading mostly to the formation of its dismutation product H_2O_2 at low NO levels (~80%), while NO mostly decays by reaction 3, leading to $ONOO^-$ formation. At higher NO levels, $ONOO^-$ formation increases, leading to the nitration of proteins and lipids that represent key biologically relevant redox signaling and injury events (42). From this view, the modulation of the activity of mtNOS and the resulting superoxide anion-derived production, H_2O_2, and peroxynitrite yield participate significantly in life processes. In the last decades, cumulative evidence has shown that H_2O_2 and the related oxidative stress level play significant roles in the activation of signaling pathways to control cell proliferation, differentiation, apoptosis, and senescence. Furthermore, redox status is clearly related to the activity of growth factors and to cell transformation and cancer. Our underlying proposal is that grading expression and activity of mtNOS and the resultant matrix NO concentration modulates H_2O_2 and oxidative stress, mediating the transmission of many intracellular signals, including those related to cell growth and transformation or to apoptosis (43).

mtNOS, Redox State, and Cell Signaling

It has long been estimated that low levels of reactive oxygen and nitrogen species can regulate the rate of proliferation (44, 45). Most cells in adult tissues are maintained in a quiescent state and reenter the cell cycle and resume proliferation only in response to tissue injury or to replace cells lost during normal tissue function (46). Cell cycle transition from G0 to G1 is mediated by cyclin-dependent kinases that are regulated by redox-sensitive cell signaling and gene expression cascades. In this context, the modulation of reactive oxygen and nitrogen species levels will determine signaling decisions between proliferation, arrest, and apoptosis.

NO and H_2O_2 are both involved in the modulation of mitogen-activated protein kinases (MAPKs) and cyclin D1. MAPKs, including stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38 MAPK, and extracellular signal–related kinases (ERK), are believed to be redox-dependent biomolecules that modulate cell proliferation, survival, and apoptosis (47). ERKs stimulate cell proliferation and induction of active cyclin D1 while p38 SAPK, transcriptionally downregulates cyclin D1.

Proliferating mammalian cells have different responses to oxidative stress level, likewise depending on the stress level (48). During rat liver development, we observed that the regulation of MAPK cascades was related to the modulation of mtNOS and subsequent redox changes in the sequence of proliferating to quiescent cell stages (Fig. 3) (26). Proliferating phenotypes (corresponding to Embryonic Days 17–19 and Postnatal Day 2) were characterized by very low levels of mtNOS activity and expression (almost not detectable by Western blot analysis), with a resulting NO-dependent H_2O_2 steady-state concentration ($[H_2O_2]_{ss}$) of 10^{-11} to 10^{-12} *M*, and high cyclin D1 expression

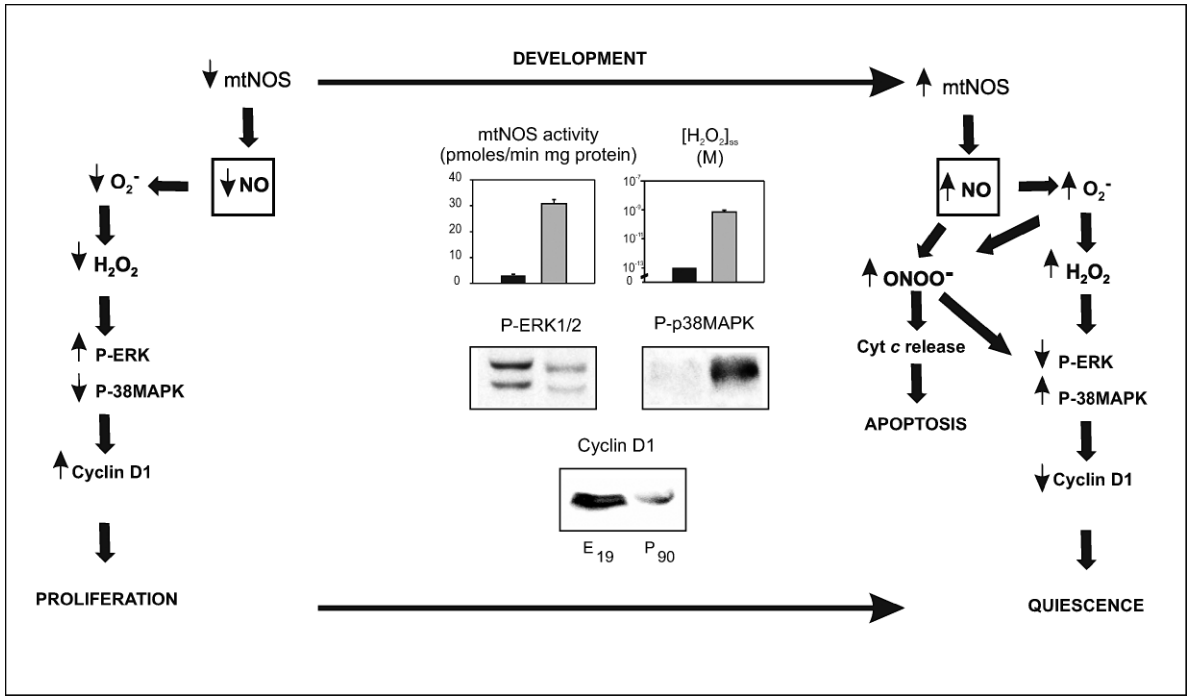

Figure 3. mtNOS and cell signaling. Scheme of the mechanism of transition of proliferating to quiescence stages during rat liver development. Inside the scheme, comparative mtNOS activity and H_2O_2 steady-state concentration measurements and calculations from embryonic and adult mitochondria are included with representative Western blots of liver homogenates with antibodies directed to phosphorylated MAPKs: ERK 1/2 and p38, and cyclin D1. E19 represents liver samples harvested from embryos at 19 days of development and P90 from adult rats (Postnatal Day 90).

associated with high ERK 1/2 and low p38 MAPK activities. In contrast, quiescent phenotypes presented an opposite pattern with NO-dependent $[H_2O_2]_{ss}$ of $\sim 10^{-9}$ *M*. These differences are sustained by the lower mitochondrial number per gram of liver tissue in proliferating tissues and the paralleled lower content of respiratory complexes and antioxidant enzyme activities.

Moreover, isolated hepatocyte proliferation rate may be also modulated by NOS inhibitors and H_2O_2 scavengers, or by MAPK inhibitors or stimulators in cell cultures (26). In this context, the synchronized increase of mitochondrial activities, mtNOS, and $[H_2O_2]_{ss}$ operate on the balance of signaling pathways to drive transition from proliferation to quiescence in rat liver development (36). In accord, an increase of mtNOS and $[H_2O_2]_{ss}$ follows rat brain and cerebellum development at the phase of synaptic plasticity (12). The mitochondrial response is consistent with the arrest of neuroblast proliferation and with apoptosis, the two components of structural synaptic plasticity.

Differential effects of H_2O_2 are also detected in transformed cells. Our group reported increased proliferation in tumor lung cells P07 and mammary LMM3 cell lines at 1 μM H_2O_2, whereas cells became arrested without apoptosis at 50–100 μM H_2O_2 (49); these effects were abolished by coincubation with catalase inhibitor 3-amino-1,2,4-triazole (ATZ). In addition, mtNOS expression is reduced in some tumor cell lines, like M3 and MM3 mammary tumors, while in others, like P07 lung tumor cells, expression is high, but mtNOS-specific activity is markedly lower than in normal tissues. Accordingly, mitochondrial H_2O_2 production was significantly lower than control mitochondria as we have observed in embryonic tissues. In this context, decreased mitochondrial oxidative phosphorylation, defective tumor mtNOS, and low NO-dependent H_2O_2 may be a platform to link persistent tumor growth to embryonic behavior.

A possible role of increased NO production by mtNOS in the setting of the death program was reported in early rat thymocyte apoptosis, associated with a loss of mitochondrial respiratory control and the release of cytochrome *c* (50), and in mitochondria-dependent follicle apoptosis during the ovarian cycle (51).

Pharmacologic induction of oxidative stress and apoptosis by tamoxifen (an anticancer drug) has been ascribed to increased mitochondrial Ca^{2+} concentration and concomitant mtNOS activation in rat liver and human breast cancer MCF-7 cells (52). Moreover, arachidonic metabolite 12(S)-hydroxyeicosatetraenoic acid (12-HETE), which is involved in pathologic conditions associated with mitochondria and oxidative stress, induces mtNOS activation and formation of peroxynitrite and subsequent cytochrome *c* release and apoptosis (53). Similarly, photodynamic therapy in combination with a photosensitizer results in a significant increase in intracellular ROS, mtNOS activation, cytochrome *c* release, and apoptotic death, attenuated by NO and singlet oxygen scavengers (54).

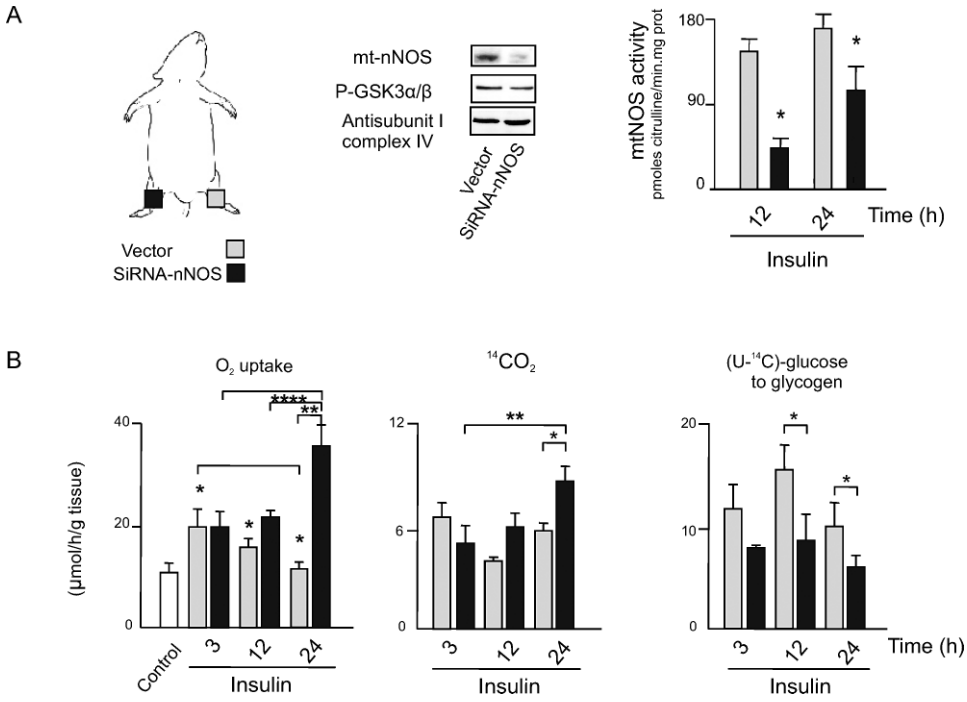

Figure 4. mtNOS and metabolic adaptation. (A) Representative Western blot of disrupted and normal mtNOS and mtNOS activities of mitochondria isolated from right and left gastrocnemius of the same animal, 36 hrs after electroporation of, respectively, 10 μg of siRNA nNOS or empty pRNAT-U6.1/Neo vector to muscle. Insulin was administered at appropriate times. (B) Metabolic studies including oxygen uptake, glucose oxidation to CO_2, and glycogen synthesis were performed at the same conditions as in A. * $P < 0.05$ of treatments vs. controls or between nNOS silenced and not-silenced muscles; ** $P < 0.05$ between different times of insulin administration. This figure was reproduced in part from the article by Finocchietto *et al.* in PLoS ONE 3(3):e1749 (19), with permission of the author.

In this context, induction of apoptosis may require fine biochemical interplay between oxygen and nitrogen species; the mechanism could be important in the step control and elimination of transformed cells (55).

Modulation of mtNOS by Hormones: Thyroid Hormones and Insulin

Thyroid Hormones. Thyroid status is crucial for energy homeostasis, and its physiologic role in growth and cell differentiation has been extensively studied. Thyroid hormone effects are exerted on mitochondria of specific target tissues, like liver and skeletal muscle, while oxidative metabolism of other organs, like brain, are not affected (56) Thyroid hormones modulate respiratory functions at two levels: (i) modifying mitochondrial inner membrane composition and permeability and (ii) influencing respiratory gene expression. Thyroid status affects mitochondrial cytochrome content. Particularly, cytochrome a–a_3 is strongly regulated; in hypothyroidism, it is decreased by 80%, whereas in hyperthyroidism, it is increased by 130% (57). Changes in properties and composition of mitochondrial membranes, particularly in cardiolipin content, lead to variations of redox enzyme activities (58, 59) and in proton leak (60).

Considering NO effects on O_2 uptake, we analyzed the effects of thyroid status on mtNOS content (8, 24). At low levels of 3,3′,5 triiodothyronine (T_3) in hypothyroidism, nNOS mRNA increased by threefold and nNOS translocation to mitochondria was favored with concomitant increase of mtNOS expression and activity.

Two main effects emerged by the mitochondrial confinement of nNOS in hypothyroidism. First, decreased mitochondrial O_2 consumption was more sensitive to L-Arg and to the NOS inhibitor N^G monomethyl L-arginine (L-NMMA), indicating the modulation of O_2 uptake by mtNOS. Second, high matrix NO resulted in high O_2^- and H_2O_2 yields and to formation of peroxynitrite. Mitochondrial redox contribution to the activation of MAPK cascades was also confirmed in the hypothyroid model. Low T_3-dependent mtNOS activation resulted in high oxidant production (H_2O_2 and peroxynitrite) with concomitant activation of p38MAPK and inactivation of ERK 1/2. As shown before, this MAPK pattern is consistent with cell cycle arrest and inhibition of cell proliferation, a hallmark of hypothyroidism. A similar effect of an NOS inhibitor (N^{ω}-nitro-L-arginine methyl esther, L-NAME) or T_3 replacement in tuning hypothyroid cell signaling back to control status indicates that differential MAPK activation and cyclin D1 expression should not depend on thyroid hormone themselves, but on the relative production of mitochondrial NO, hydrogen peroxide, and peroxynitrite, at the different T_3 levels.

In addition, an extensive nitration of mitochondrial complex I proteins in this model was associated with a

markedly reduced rate of electron transfer to ubiquinol acceptor, which contributes to perpetuating the process (33). Complex I derangement and decreased basal metabolic rate were reverted by previous administration of L-NAME to the hypothyroid rats. These findings suggest that most O_2 consumption inhibition in hypothyroidism is the consequence of complex I inhibition by NO-$ONOO^-$ overproduced by increased translocated nNOS (mtNOS). It is interesting that T_3 deficiency stimulates nNOS gene expression, which suggests the existence of a tonic gene inhibition relying on the effects of T_3 receptor dimers on the transcriptional machinery.

The notion that spatial confinement is essential to nNOS signaling is supported here by differential modulation of O_2 uptake and nNOS distribution, depending on thyroid status; this notion could be extended to other cell-adaptive responses.

Insulin Metabolic regulation is largely dependent on mitochondria, which play an important role in energy homeostasis by metabolizing nutrients and producing ATP and heat. Genetic and environmental factors, including exercise, diet, aging, and stress, affect both mitochondrial function and insulin sensitivity. Importantly, mitochondrial dysfunction has been associated with insulin resistance in skeletal muscle, as well as in other tissues, including liver, fat, heart, vessels, and pancreas.

It was recently reported that in the metabolic syndrome with hyperinsulinemia, mitochondrial dysfunction facilitates muscle fat accumulation (61) and accelerates its progression. Considering NO as a typical mitochondrial modulator, we previously demonstrated that insulin-operated signaling pathways modulate mitochondrial respiration *via* NO to alternatively release complete glucose oxidation or to drive glucose storage to glycogen. We confirmed that NO produced by translocated nNOS (mtNOS) is the insulin-signaling molecule that controls mitochondrial oxygen utilization (19).

Accumulating evidence implicates the insulin-activated, phosphatidylinositol-3′-kinase (PI3K)–dependent Ser/Thr Akt/protein kinase B (PKB) as a regulator of glucose transport, glycolysis, and suppression of gluconeogenesis, cell survival, and cell-cycle progression (62). In mice mitochondria, mtNOS is phosphorylated in an Akt-sensitive domain at Ser^{1412}. Recent reports suggest that Akt1 and Akt2 have distinct functions in cellular regulation and metabolism, with Akt 2 performing an important metabolic function (63). In this way, we confirmed that insulin determines *in situ* phosphorylation of mtNOS by Akt2 with an hyperinsulinemic-normoglycemic noninvasive clamp model, showing that this effect relies on p-Akt2–selective phosphorylation of mitochondrial nNOS after the kinase translocation to the organelles (19). A rapid translocation of p-Akt2 to the mitochondria and the consequent phosphorylation of target GSK-3α/β (64) by insulin-phosphatidyl inositol 3-kinase (PI3K), resulting in nNOS activation (+4–8 fold), without protein expression changes, high NO yield, and the concomitant decrease of mitochondrial oxygen uptake and resting metabolic rate (–25% to –60%).

By disrupting mtNOS, the direct comparative electroporation of siRNA nNOS or empty vector on gastrocnemius muscle in the legs of the same animal confirmed the *in vivo* insulin metabolic effects leading to higher oxygen uptake and complete glucose oxidation and restricted glycogen synthesis by one half (Fig. 4). However, silencing nNOS does not appear to modify glycogen synthetase activity or muscle glucose uptake *per se*, and thus, the glycogen synthesis rate is presumed to mainly rely on the availability of substrate.

It is surmised that modulation of O_2 uptake by mitochondrial NO serves a variety of biological purposes, such as adjusting the oxidative rate to O_2 available or setting up the energy threshold for cell proliferation or apoptosis.

Otherwise, persistent activation of mtNOS and increase of mitochondrial NO likewise contribute to mitochondrial damage. In this context, a number of different NO-based mitochondrial mechanisms can contribute to insulin resistance as well. First, a reduction in ATP levels due to inhibition of electron transfer can restrict the phosphorylation of insulin receptor substances (IRS) and the translocation of GLUT4 to the cell membrane. Second, accumulation of NADH due to NO-dependent complex I inhibition (63) negatively modulates glyceraldehide-3–phosphate dehydrogenase, citrate synthase, and α-ketoglutarate dehydrogenase. Finally, increased muscle glycogen and fat deposition can contribute *per se* to resistance and to increased insulin requirements (64).

It is believed that mtNOS activation leads to insulin resistance and mitochondrial dysfunction and represents a common pathophysiologic etiology for many chronic diseases.

1. Brown GC. Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett 369:136–139, 1995.
2. Poderoso JJ, Carreras MC, Lisdero C, Riobó N, Schöpfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch Biochem Biophys 328:85–92, 1996.
3. Borutaite V, Brown GC. Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem J 315:295–299, 1996.
4. Michel T, Feron O. Nitric oxide synthases: which, where, why? J Clin Invest 100:2146–2152, 1997.
5. Bates TE, Loesch A, Burnstock G, Clark JB. Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. Biochem Biophys Res Commun 213:896–900, 1995.
6. Ghafourifar P, Richter C. Nitric oxide synthase activity in mitochondria. FEBS Lett 418:291–296, 1997.
7. Giulivi C, Poderoso JJ, Boveris A. Production of nitric oxide by mitochondria. J Biol Chem 273:11038–11043, 1998.
8. Carreras MC, Peralta JG, Converso DP, Finocchietto PV, Rebagliati I, Zaninovich AA, Poderoso JJ. Mitochondrial nitric oxide synthase is a

final effector in the thyroid-dependent modulation of oxygen uptake. Am J Physiol 281:H2282–H2288, 2001.
9. Tatoyan A, Giulivi C. Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. J Biol Chem 273:11044–11048, 1998.
10. Bates TE, Loesch A, Burnstock G, Clark JB. Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation? Biochem Biophys Res Commun 218:40–44, 1996.
11. Koivisto A, Matthias A, Bronnkov G, Nedergaard J. Kinetics of the inhibition of mitochondrial respiration by NO. FEBS Lett 417:75–80, 1997.
12. Riobó N, Melani M, Sanjuán N, Carreras MC, Cadenas E, Poderoso JJ. The modulation of mitochondrial nitric oxide synthase activity in rat brain development. J Biol Chem 277:42447–42455, 2002.
13. Frandsen U, López-Figueroa M, Hellsten Y. Localization of nitric oxide synthase in human skeletal muscle. Biochem Biophys Res Commun 227:88–93, 1996.
14. Carreras MC, Melani M, Riobó N, Converso DP, Gatto EM, Poderoso JJ. Neuronal nitric oxide synthases in brain and extraneural tissues. Methods Enzymol 359:413–423, 2002.
15. Valdez LB, Zaobornyj T, Alvarez S, Bustamante J, Costa LE, Boveris A. Heart mitochondrial nitric oxide synthase. Effects of hypoxia and aging. Mol Aspects Med 25:49–59, 2004.
16. Elfering SL, Sarkela TM, Giulivi C. Biochemistry of mitochondrial nitric-oxide synthase. J Biol Chem 277:38079–38088, 2002.
17. Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, de Groat WC, Peterson J. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. Proc Natl Acad Sci U S A 98:14126–14131, 2001.
18. Carreras MC, Franco MC, Molinas MF, González AS, Finocchietto PV, Converso DP, Poderoso JJ. Neuronal nitric oxide synthase trafficking into mitochondria. Free Radic Biol Med 45(S1):S111, 2008.
19. Finocchietto PV, Barreyro F, Holod S, Peralta JG, Franco MC, Méndez C, Converso DP, Estévez AG, Carreras MC, Poderoso JJ. Control of muscle mitochondria by insulin entails activation of Akt2-mtNOS pathway: implications for the metabolic syndrome. PLoS ONE 3(3): e1749, 2008.
20. Lisdero CL, Carreras MC, Meulemans A, Melani M, Aubier M, Boczkowski J, Poderoso JJ. The mitochondrial interplay of ubiquinol and nitric oxide in endotoxemia. Methods Enzymol 382:67–81, 2004.
21. López LC, Escames G, Tapias V, Utrilla P, León J, Acuña-Castroviejo D. Identification of an inducible nitric oxide synthase in diaphragm mitochondria from septic mice: its relation with mitochondrial dysfunction and prevention by melatonin. Int J Biochem Cell Biol 38:267–278, 2006.
22. Averna M, Stifanese R, De Tullio R, Salamino F, Bertuccio M, Pontremoli S, Melloni E. Proteolytic degradation of nitric oxide synthase isoforms by calpain is modulated by the expression levels of HSP90. FEBS J 274:6116–6127, 2007.
23. Takahashi S, Mendelsohn ME. Synergistic activation of endothelial nitric-oxide synthase (eNOS) by HSP90 and Akt: calcium-independent eNOS activation involves formation of an HSP90-Akt-CaM–bound eNOS complex. J Biol Chem 278:30821–30827, 2003.
24. Franco MC, Antico Arciuch VG, Peralta JG, Galli S, Levisman D, López LM, Romorini L, Poderoso JJ, Carreras MC. Hypothyroid phenotype is contributed by mitochondrial complex I inactivation due to translocated neuronal nitric oxide synthase. J Biol Chem 281:4779–4786, 2006.
25. Tay YM, Lim KS, Sheu FS, Jenner A, Whiteman M, Wong KP, Halliwell B. Do mitochondria make nitric oxide? No? Free Radic Res 38:591–599, 2004.
26. Carreras MC, Converso DP, Lorenti AS, Barbich MR, Levisman DM, Jaitovich A, Antico Arciuch VG, Galli S, Poderoso JJ. Mitochondrial nitric oxide synthase drives redox signals from proliferation and quiescence in rat liver development. Hepatology 40:157–166. 2004.
27. Peralta JG, Finocchietto PV, Converso D, Schopfer F, Carreras MC, Poderoso JJ. Modulation of mitochondrial nitric oxide synthase and energy expenditure in rats during cold acclimation. Am J Physiol Heart Circ Physiol 284:H2375–H2383, 1993.
28. Parihar MS, Parihar A, Chen Z, Nazarewicz R, Ghafourifar P. mAtNOS1 regulates mitochondrial functions and apoptosis of human neuroblastoma cells. Biochim Biophys Acta 1780:921–926, 2008.
29. Poderoso JJ. The formation of peroxynitrite in the applied physiology of mitochondrial nitric oxide. Arch Biochem Biophys 484:214–220, 2009.
30. Antunes F, Cadenas E. The mechanism of cytochrome c inhibition by nitric oxide. Frontiers Biosci 12:975–985, 2007.
31. Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AHV. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. FEBS Lett 345:50–54, 1994.
32. Brown GC. Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett 369:136–139, 1995.
33. Poderoso JJ, Lisdero C, Schöpfer F, Riobó N, Carreras MC, Cadenas E, Boveris A. The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol. J Biol Chem 274: 37709–37716, 1999.
34. Cassina A, Radi R. Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch Biochem Biophys 328:309–316, 1996.
35. Brookes PS, Kraus DW, Shiva S, Doeller JE, Barone MC, Patel RP, Lancaster JR Jr, Darley-Usmar V. Control of mitochondrial respiration by NO, effects of low oxygen and respiratory state. J Biol Chem 278: 31603–31609, 2003.
36. Poderoso JJ, Peralta JG, Lisdero C, Carreras MC, Radisic M, Schöpfer F, Cadenas E, Boveris A. Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. Am J Physiol Cell Physiol 274:112–119, 1998.
37. Carreras MC, Franco MC, Peralta JG, Poderoso JJ. Nitric oxide, complex I, and the modulation of mitochondrial reactive species in biology and disease. Mol Asp Med 25:125–139, 2004.
38. Poderoso JJ, Carreras MC, Schöpfer F, Lisdero C, Riobó N, Giulivi C, Boveris A, Boveris AA, Cadenas E. The reaction of nitric oxide with ubiquinol: kinetic properties and biological significance. Free Rad Biol Med 26:925–935, 1999.
39. Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci 28:57–87, 2005.
40. Boveris A, Oshino N, Chance B. The cellular production of hydrogen peroxide. Biochem J 128:617–630, 1972.
41. Boveris A, Poderoso JJ. Regulation of oxygen uptake by nitric oxide. In: Ignarro L, Ed. Nitric Oxide. Biology and Pathobiology, San Diego: Academic Press, pp355–368, 2000.
42. Rubbo H, Radi R. Protein and lipid nitration: role in redox signaling and injury. Biochim Biophys Acta 1780:1318–1324, 2008.
43. Carreras MC, Poderoso JJ. Mitochondrial NO and cell-integrated responses. Am J Physiol Cell Physiol 292:C1569–C1580, 2007.
44. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Rad Biol Med 18:775–794, 1995.
45. Villalobo A. Nitric oxide and cell proliferation. FEBS J 273:2329–2344, 2006.
46. Burch PM, Heintz NH. (2005) Redox regulation of cell-cycle re-entry: cyclin D1 as a primary target for the mitogenic effects of reactive oxygen and nitrogen species. Antioxid Redox Signal 7:741–751, 2006.
47. Chang L, Karin M. Mammalian MAP kinase signaling cascades. Nature 410:37–40, 2001.
48. Davies KJA. The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress. IUBMB Life 48:41–47, 2000.

49. Galli S, Labato M, Bal de Kier Joffè E, Carreras MC, Poderoso JJ. Decreased mitochondrial nitric oxide synthase activity and hydrogen peroxide relate persistent tumoral proliferation to embryonic behavior. Cancer Res 60:6073–6077, 2003.
50. Bustamante J, Bersier G, Romero M, Badin RA, Boveris A. Nitric oxide production and mitochondrial dysfunction during rat thymocyte apoptosis. Arch Bioch Biophys 376:239–247, 2000.
51. Navarro A, Torrejon R. Role of nitric oxide on mitochondrial biogenesis during the ovarian cycle. Front Biosci 12:1164–1173, 2007.
52. Nazarewicz RR, Zenebe WJ, Parihar A, Larson SK, Alidema E, Choi J, Ghafourifar P. Tamoxifen induces oxidative stress and mitochondrial apoptosis via stimulating mitochondrial nitric oxide synthase. Cancer Res 67:1282–1290, 2007.
53. Nazarewicz RR, Zenebe WJ, Parihar A, Parihar MS, Vaccaro M, Rink C, Sen CK, Ghafourifar P. 12(S)-Hydroperoxyeicosatetraenoic acid (12-HETE) increases mitochondrial nitric oxide by increasing intramitochondrial calcium. Arch Biochem Biophys 468:114–120, 2007.
54. Lu Z, Tao Y, Zhou Z, Zhang J, Li C, Ou L, Zhao B. Mitochondrial reactive oxygen species and nitric oxide–mediated cancer cell apoptosis in 2-butylamino-2-demethoxyhypocrellin B photodynamic treatment. Free Radic Biol Med 41:1590–1605, 2006.
55. Bauer G. Reactive oxygen and nitrogen species: efficient, selective, and interactive signals during intercellular induction of apoptosis. Anticancer Res 20:4115–4139, 2000.
56. Reitman ML, He Y, Gong D-W. Thyroid hormone and other regulators of uncoupling proteins. Int J Obes Relat Metab Disord 23:S56–S59, 1999.
57. Nogueira V, Walter L, Av'eret N, Fontaine E, Rigoulet M, Leverve XM. Thyroid status is a key regulator of both flux and efficiency of oxidative phosphorylation in rat hepatocytes. J Bioenerg Biomembr 34: 55–66, 2002.
58. Hulbert AJ, Augee ML, Raison JK. The influence of thyroid hormones on the structure and function of mitochondrial membranes. Biochim Biophys Acta 455:597–601, 1976.
59. Schlame M, Hostetler KY. Cardiolipin synthase from mammalian mitochondria. Biochim Biophys Acta 1348:207–213, 1997.
60. Brand MD, Chien L-F, Ainscow EK, Rolfe DFS, Porter RK. The causes and functions of mitochondrial proton leak. Biochim Biophys Acta 1187:132–139, 1994.
61. Petersen KF. Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 350:664–671, 2004.
62. Whiteman EL, Cho H, Birnbaum MJ. Role of Akt protein kinase β in metabolism. Trends Endocrinol Metab 13:444–450, 2002.
63. Bellacosa A, Testa JR, Moore R, Larve L. A portrait of Akt kinases. Cancer Biol Ther 3:268–275, 2004.
64. Van de Sande T, De Schrijver E, Heyns W, Verhoeven G, Swinnen JV. Role of the phosphatidylinositol 3-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. Cancer Res 62:642–646, 2002.
65. Mohr S, Hallak H, Boitte A, Lapetina EG, Brüne B. Nitric oxide–induced S-glutathionylation and inactivation of glyceraldehydes-3-phosphate dehydrogenase. J Biol Chem 274:9427–9430, 1999.
66. Rask-Madsen C, King GL. Mechanism of disease: endothelial dysfunction in insulin resistance and diabetes. Nature 3:46–56, 2007.