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The multifaceted role of nitric oxide synthases in mitochondrial biogenesis and cell differentiation

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Nitric oxide (NO) is physiologically synthetized by a family of enzymes called NO synthases (NOSs). NO is a pleiotropic second messenger having a fundamental role in several cellular processes including cell differentiation. Being a high reactive molecule, NO must be synthetized in close proximity to the effector/target. For this reason, the subcellular localization of NOSs is tightly regulated by different post-translation mechanisms. Recently, in murine C2C12 myoblasts, we have demonstrated that mitochondrial biogenesis, an essential event for cell differentiation, can be effective only if the site of NO production is located at nuclear level, where NO favors the CREB-dependent expression of PGC-1α gene. The increase of NO flux in nuclei is elicited by the up-regulation and redistribution of neuronal NOS (nNOS) toward nuclei.

Herein we show that an upregulation of endothelial NOS (eNOS) occurs during adipocyte differentiation in 3T3-L1 cells. However, differently to differentiating myocytes, a concomitant redistribution of eNOS toward nuclei was not detected. We also observed that, upon treatment with the NO synthesis inhibitor L-NAME, mitochondrial biogenesis as well as triglyceride accumulation that normally occurs during adipogenesis were not impeded. The absence of eNOS in nuclei together with the ineffectiveness of L-NAME suggest that, at least during 3T3-L1 differentiation, NO is not fundamental for the induction of mitochondrial biogenesis and adipogenesis.

Keywords: adipogenesis, adipose tissue, myogenesis, mitochondria, skeletal muscle

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Among these a cardinal role is exerted by nitric oxide (NO), a second messenger produced physiologically by a family of enzymes called NO synthases (NOSs) by oxidation of a guanidino nitrogen of L-arginine into L-citrulline and NO.[1] Three distinct NOS isoforms exists, i.e. nNOS or NOS1 (neuronal NOS), iNOS or NOS2 (inducible NOS) and eNOS or NOS3 (endothelial NOS) that are encoded by 3 different genes on different chromosomes, and exhibit about 60% identity with each other and with cytochrome P450 enzymes.[2] To synthesize NO, the NOS isoforms utilize several cofactors and co-substrates including molecular oxygen, NADPH, tetrahydrobiopterin, flavin mononucleotide, flavin adenine dinucleotide, heme, and calmodulin. iNOS is mainly expressed in immune cells during the inflammatory response. eNOS and nNOS are expressed constitutively and their activity is regulated by calcium/calmodulin and by multiple post-translational mechanisms among which protein-protein interaction and subcellular redistribution is included.[3]

Even though NO has been widely recognized to modulate cell differentiation, whether it has a negative or positive role in this process is still a debated matter of research. NO generally promotes angiogenic differentiation both under physiological and pathological conditions.[4] In nervous system, NO has a dual role in modulating neurogenesis during development and in adulthood after brain injury. The production of NO by nNOS appears to inhibit neurogenesis, whereas NO synthesized by eNOS and iNOS stimulate neurogenesis.[5]

nNOS is the predominant NOS isoform in skeletal muscle cells and has a

A very intricate network of signaling pathways drives cell differentiation.

central role in regulating blood flow and contractile activity as well as myogenesis.[1] In differentiating murine C2C12 myoblasts, we have demonstrated that increased localization of nNOS to the nuclear envelope is mandatory to activate the mitochondrial biogenesis and myogenic program.[6] In particular, the enhancement of NO synthesis in close proximity to nuclei is crucial for activating the cAMP response element-binding protein (CREB) via S-nitrosylation. This post-translational modification allows CREB to more efficiently engage PGC-1α promoter, up-regulate its expression and induce mitochondrial biogenesis and in turn myocyte differentiation. The redistribution of nNOS to nuclei is favored by protein-protein interaction with the nuclear-associated α-syntrophin via the nNOS PDZ domain. Interestingly, overexpressed nNOS accumulates at the nuclear level and induces mitochondrial biogenesis. Conversely, overexpression of a nNOS form lacking the PDZ domain does not localize to nucleus and, albeit fully preserving NO synthesizing activity, is not able to promote mitochondrial biogenesis.[6]

Adipose tissue is the major fat sink in the body and represents a specialized system that tightly responds to deprivation or excess of nutrients to maintain whole-body energy homeostasis.[7-9] Adipose tissue expansion physiologically occurs during aging and is dramatically accelerated in metabolic diseases reducing lifespan.[10-11] Besides nutrients, adipogenesis represents another critical determinant of adipose tissue mass.[12] In adipose tissue both eNOS and iNOS are expressed and their increased amount was observed in white adipose tissue samples of obese humans.[13-14] These findings suggest that NO can be an inducer of adipogenesis and/or an inhibitor of lipolysis. In support of an anti-lipolytic effect of NO, it has been demonstrated that the blockage of NO production through the *pan*-NOSs inhibitor L-NAME increases triglyceride hydrolysis, while challenge with NO donors inhibits lipolysis in human white adipocytes.[15] In brown adipose tissue, it has been demonstrated that NO produced by eNOS stimulates pre-adipocytes differentiation. Indeed, chronic treatment with NO donors favors adipogenesis, while inhibition of NO production through L-NAME in primary brown adipocyte significantly blunts differentiation.[16] Mitochondrial biogenesis is an essential event occurring during cell differentiation.[17-19] Coherently, mitochondrial biogenesis is also inhibited during differentiation of brown adipocytes upon treatment with L-NAME.[16]

Whether NO modulates adipogenesis in white adipocytes is still controversial. It has been proposed that expansion of adipose tissue observed in obese individuals could be due to iNOS-mediated NO production typical of the chronic inflammatory state.[20] In line with this evidence, chronic treatment with a NO donor in human pre-adipocytes significantly enhances adipogenesis.[21] Moreover, it has been shown that NO produced by eNOS promotes differentiation in rat white pre-adipocytes.[22] A commonly used model to study adipogenesis in white adipose tissue is represented by mouse 3T3-L1 pre-adipocytes.[23] 3T3-L1 cells treated with a NO donor loss the capability to fully differentiate as NO inhibits the activity of the adipogenic transcription factor PPARγ.[24]

Here we show that, during 3T3-L1 differentiation, mitochondrial biogenesis is effective after day 2. Indeed, a significant increase of mitochondrial mass was observed starting at Day 4. At the end of differentiation (Day 8) mitochondrial mass was markedly increased (**Fig. 1A**). Coherently, PGC-1α and TFAM (**Fig. 1B**), which represent well-known markers of mitochondrial biogenesis, were significantly up-regulated in differentiated (Day 8) with respect to undifferentiated cells (day 0). eNOS protein content (**Fig. 1B**) and activity (**Fig. 1C**) was increased as well, while iNOS protein was not detectable either prior or after differentiation (data not shown). NO has been recognized to modulate cell proliferation in adipocytes precursors.[16] Up to day 2, 3T3-L1 cells undergo mitotic clonal expansion and do not show an efficient induction of mitochondrial biogenesis (**Fig. 1A**). Thus, to investigate the role of NO in the differentiation process, we added L-NAME (1 mM) starting at Day 2 up to Day 8 of differentiation. L-NAME has not impact on the protein content of eNOS (**Fig. 1B**) but efficiently inhibits NO production (**Fig. 1C**). However, L-NAME was able to constrain neither complete adipocyte differentiation nor mitochondrial biogenesis (**Fig. 1E**), as demonstrated by unchanged triglyceride content (**Fig. 1D**) and unaltered levels of PGC-1α and TFAM protein (**Fig. 1B**) as well as enhancement of mitochondrial mass (**Fig. 1E**). These results suggest that, at least in this *in vitro* murine model of adipogenesis, endogenous NO production is not involved in differentiation and mitochondrial biogenesis.

Interestingly, both eNOS and iNOS were found localized and metabolically active in nuclei of brown adipocytes.[25] Cold exposure or β(3)-adrenergic agonist treatment significantly increased nuclear eNOS and iNOS expression and activities, suggesting the existence of a noradrenaline-modulated functional NOS system in the nucleus of brown adipocytes.[25] Conversely, in 3T3-L1 adipocytes we have not observed eNOS in nuclear extracts (**Fig. 1B**). The data we obtained in 3T3-L1 adipocytes are in agreement with what observed by Nisoli and co-workers, who demonstrated that under resting condition $eNOS^{-/-}$ mice did not show any effect on white adipose tissue mass as well as mitochondrial number compared to wild type mice.[26] However, since we observed an up-regulation of eNOS in differentiated adipocytes, it can be assumed that the NO overproduction must necessarily have a role within adipocytes that surely merits more deep investigation. It has been recently demonstrated that $eNOS^{-/-}$ mice are not able to metabolically adapt to exercise training. In particular, eNOS appears to be involved in the induction of mitochondrial biogenesis in subcutaneous adipose tissue of trained mice.[27] It has been also observed that the inhibition of eNOS activity in differentiated 3T3-L1 adipocytes impairs mitochondrial biogenesis elicited by lipoamide treatment.[28] On the basis of overall these findings it can be speculated that NO endogenously produced by basal eNOS in white adipocytes is not involved in the modulation of adipogenesis-related mitochondrial biogenesis and adipogenesis itself. Therefore, a hypothesis could be that in mature adipocytes NO generated by eNOS may have a role in stimulating

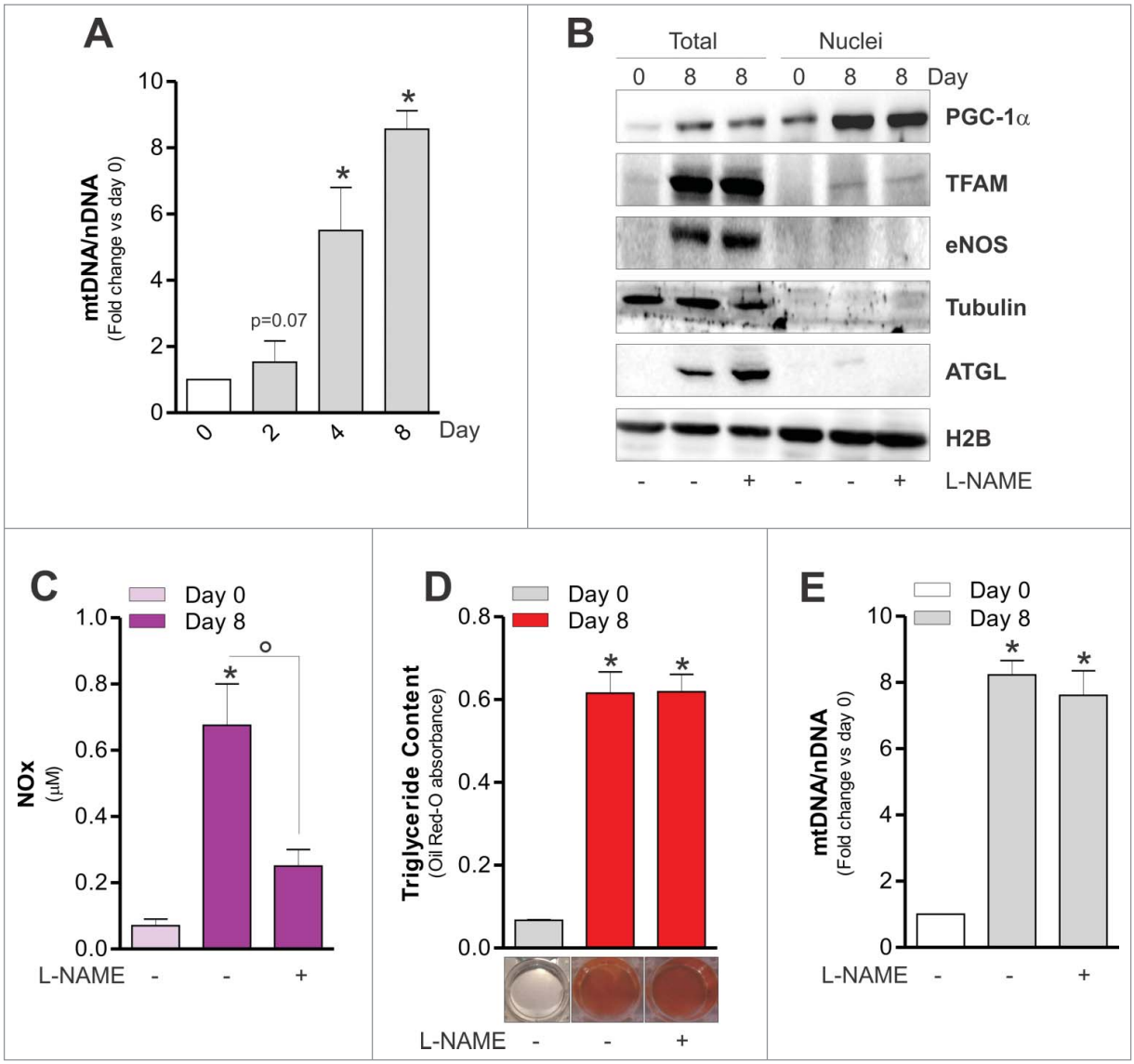

Figure 1. Inhibition of NO production does not affect mitochondrial biogenesis and adipogenesis in 3T3-L1 cells. (**A**) Mitochondrial mass was monitored by assaying the level of mtDNA through qPCR analysis of D-Loop. Data are expressed as means of fold changes of mtDNA/nDNA with respect to undifferentiated cells (Day 0) ± SD (n = 4, *$P < 0.001$ *vs* Day 0). (**B**). PGC-1α, TFAM, eNOS and ATGL were detected by western blot analysis in total and nuclear extracts. L-NAME (1 mM) was added in culture medium after mitotic clonal expansion (Day 2) and maintained up to the end of differentiation (Day 8). Tubulin and H2B were used as loading and to assess the purity for the nuclear fraction. ATGL was used as marker of adipocyte differentiation. (**C**) NO production was evaluated by measuring nitrites and nitrates (NO_x) released in culture medium by Griess reaction. Data are expressed as means ± SD (n = 4, *$P < 0.001$ *vs* Day 0; °$P < 0.001$ *vs* L-NAME-untreated Day 8). (**D**) Triglyceride content was determined by measuring the absorbance of eluted Oil red-O. Cells were treated with L-NAME as described in (**B**). Data are expressed as means ± SD (n = 4, *$P < 0.001$ vs Day 0). (**E**) Mitochondrial mass was assessed as described in (**A**). Cells were treated with L-NAME as described in (**B**). Data are expressed as means of fold changes of mtDNA/nDNA with respect to undifferentiated cells (Day 0) ± SD (n = 4, *$P < 0.001$ *vs* Day 0).

the metabolic activity of mitochondria and may be involved in the white-to-brown transition of adipose tissue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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