Neuronal nitric oxide synthase controls enzyme activity pattern of mitochondria and lipid metabolism

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ABSTRACT

Mitochondria are affected by endogenous nitric oxide (NO). Besides effects of NO on mitochondrial enzymes and the stimulation of mitochondrial H₂O₂ production, a NO-dependent increase in mitochondrial biogenesis in several tissues has been reported. It is still obscure whether NO generated by one specific or different NO synthase (NOS) isoforms determine such effects. Therefore, we analyzed the amount of mitochondria, respiratory chain enzyme complexes, and citrate synthase in the brain, muscle, heart, kidney, and liver by comparing wild-type (WT) mice and mice lacking the neuronal nitric oxide synthase isoform (nNOS-KO). Our results show that the activities of NADH:cytochrome c oxidoreductase and succinate cytochrome c oxidoreductase differ between WT and nNOS-KO mice. However, similar quantities of mitochondria were found in the homogenates of tissues in WT and nNOS-KO animals. Most impressive, higher activities and protein of citrate synthase were found in the brain, muscle, heart, kidney, and liver of nNOS-KO mice. Additionally, higher contents of fatty acid synthase and lipids were determined in the livers of nNOS-KO mice but not in the heart and brain. Furthermore, liver mitochondria from nNOS-KO mice consumed pyruvate at a higher rate and released more citric acid. Our data document a previously unrecognized role of endogenous NO in the regulation of lipid metabolism.

Key words: endogenous nitric oxide • citrate synthase • respiratory chain

NO is endogenously generated in mammals in the presence of oxygen by the conversion of arginine to citrulline via a family of NOS isoforms. Three distinct genes have been identified encoding the neuronal (nNOS), the endothelial (eNOS), and the inducible (iNOS) isoform of NOS (1). Several splice variants of these NOS isoforms have been characterized. In addition, mitochondrial NOS species (mtNOS) have been found in a number of tissues (2–6). It was demonstrated that the mtNOS in liver shares characteristics with the α-splice isoform of the nNOS.
The highly diffusible and hydrophobic NO performs several physiological functions including the regulation of blood flow by stimulating vasodilatation, neurotransmission, and mediation of immunoresponse. Furthermore, NO has been demonstrated to interact with mitochondria. Most widely studied is the inhibition of mitochondrial respiratory chain complexes by NO, in particular of the cytochrome c oxidase. Inhibition of this complex by NO has been shown in isolated mitochondria in cells and tissues (7–9). This effect of NO is mediated by reversible binding of NO, in competition with O2, to a binuclear heme-copper ion center. Subsequent reduction of mitochondrial O2 consumption has been interpreted as a mechanism for the regulation of O2 gradients within tissues resulting in the extension of O2 supply distal to blood vessels (10). In addition, the interaction of NO with the cytochrome c oxidase controls mitochondrial production of hydrogen peroxide (H2O2), which has been shown to be implicated in cellular redox signaling (11, 12).

Recently, promotion of mitochondrial biogenesis by NO has been reported for different tissues. Significantly lower quantities of mitochondria were found in mice lacking functional eNOS compared with WT animals (13). However, it remains unclear whether NO, which originates from NOS isoenzymes other than eNOS, can stimulate the biogenesis of mitochondria and affects the enzyme activity pattern of mitochondria in these tissues. Therefore, we used mice lacking nNOS to study the effect of endogenous NO on mitochondria. In comparison to eNOS, nNOS is differently distributed in the tissues and, therefore, specifically contributes to tissue NO levels. In this context, we studied the quantity of mitochondria, the activity of respiratory chain enzyme complexes, and the activity and the amount of protein of citrate synthase as a representative of the citrate acid cycle in the brain, skeletal muscle, heart, kidney, and liver depending on the presence of nNOS. In the tissues investigated, the lack of nNOS (nNOS-KO mouse) was without any effect on the content of mitochondria. Additionally, moderately differing activities of NADH:cytochrome c oxidoreductase and succinate cytochrome c oxidoreductase were found in comparison to WT animals. In nNOS-KO mice, significant higher tissue activities and protein contents of citrate synthase (up to 3-fold) were determined. In liver, higher levels of lipids were found in nNOS-KO mice. Further, liver mitochondria from nNOS-KO mice consumed pyruvate at a higher rate and released more citric acid into the extramitochondrial space. From our data, we conclude that NO derived from nNOS essentially regulates fatty acid synthesis as well as mitochondrial electron transport.

MATERIALS AND METHODS

Animals

All experiments were performed using 60- to 70-day-old laboratory bred homozygous nNOS knock out mice (nNOS-KO) as developed in the C57BL/6 strain (14, 15) and wild-type mice (WT) from the same background. All animals were maintained in accordance with the guidelines of the German Animal Welfare Act. The experimental protocol was approved by a review committee of the state of Sachsen-Anhalt, Germany.

Preparation of tissue homogenates

The extracted tissues were minced using small scissors and transferred into ice cold phosphate buffer solution (PBS) at pH 7.4. Subsequently, the tissues were homogenized at 4°C using a
Potter-Elvehjem glass-Teflon homogenizer performing 10 strokes at 600 rpm. After determination of protein, all samples were adjusted to identical protein content by adding adequate amounts of PBS.

**Preparation of mitochondria**

Liver mitochondria were prepared from the livers of WT mice in ice-cold medium containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, and 1% (w/v) bovine serum albumin (BSA) using a standard procedure (16). After the initial isolation, Percoll was used for purification of mitochondria from a fraction containing some endoplasmatic reticulum, Golgi apparatus, and plasma membranes. The mitochondria were well coupled, as indicated by a respiratory control index >5 with glutamate plus malate as substrates.

Mitochondria from brain and heart were isolated from the corresponding organs of WT animals in a medium containing 250 mM mannitol, 20 mM Tris, 1 mM EGTA, 1 mM EDTA, and 0.3% BSA (w/v) at pH 7.4 by using a standard procedure (17). The mitochondria were well coupled, as indicated by a respiratory control index >4 with glutamate plus malate as substrates.

**Incubation of mitochondria**

Mitochondria (0.5–1.0 mg of protein/ml) were incubated in a medium containing 10 mM KH₂PO₄, 0.5 mM EGTA, 60 mM KCl, 60 mM Tris, 110 mM mannitol, and 1 mM free Mg²⁺ at pH 7.4 and 30°C.

**Measurement of respiration**

Oxygen uptake of the mitochondria was measured at 30°C in a thermostat-controlled chamber equipped with a Clark-type electrode. For the calibration of the oxygen electrode, the oxygen content of the air-saturated incubation medium was taken to be 217 nmol/ml (18).

**Determination of NADH:cytochrome c oxidoreductase activity (EC 1.6.99.3)**

Samples of 500 µl were withdrawn from the homogenates. The mitochondria were disintegrated by sonication. A 15 µl volume (40 µg protein) was used to run the assay at 30°C as described previously (19). The reduction of cytochrome c was followed by measurement of the absorption at 550 nm with a Varian spectrophotometer (Cary 1E). The rotenone-sensitive absorption was used for quantification.

**Determination of succinate cytochrome c oxidoreductase activity (EC 1.3.5.1)**

Samples of 500 µl were withdrawn from the homogenates. The mitochondria were disintegrated by sonication. A 15 µl volume (40 µg protein) was used to run the assay at 30°C as described previously (20). The reduction of cytochrome c was followed by measurement of the absorption at 550 nm with a Varian spectrophotometer (Cary 1E). The antimycin A-sensitive absorption was used for quantification.
Western blot analysis

For immunoblotting, homogenates from the brain, muscle, heart, kidney, and liver were mixed with protein loading buffer (roti-Load 1, Carl Roth GmbH, Karlsruhe, Germany) according to the manufacturer’s procedure and placed in a heating bath (95°C) for 5 min. Proteins were separated using SDS-PAGE (gradient gels from 5 to 25%). The protein amount loaded per lane was 20 µg. After separation, the proteins were stained with Coomassie brilliant blue or transferred to nitrocellulose paper and unspecific protein binding sites were blocked with blocking buffer (Chemicon International, Hofheim, Germany). The blots were incubated overnight with a primary antibody against nNOS (polyclonal, BD Biosciences, Denderstraat, Belgium, 1:1000), cytochrome c (monoclonal, BD Biosciences, 1:200), citrate synthase (monoclonal, Chemicon International, 1:200), or fatty acid synthase (BD Biosciences, 1:200) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit and anti-mouse IgG+ peroxidase, Boehringer Mannheim GmbH, 1:10,000). Immunoreactivity was visualized using the ECL detection system (Amersham Pharmacia Biotech Lim., Buckinghamshire, UK). Additionally, β-actin detection was used to show equal sample loading.

Electron microscopy

Specimens for counting mitochondria were taken from the respective organs of three WT and three nNOS-KO mice, fixed in 0.2 M cacodylate buffer containing 2.5% glutaraldehyde, and then osmicated (2% OsO₄), dehydrated, en-bloc stained with 7% uranyl acetate, and, finally, embedded in Durcupan (ACM, Fluka/Sigma). Ultra-thin sections (50–70 nm) were mounted on Formvar-coated slot grids. Three arbitrarily selected grids from each probe were examined with a transmission electron microscope E 900 (Zeiss, Germany). Five cells (neurons in brain material, myocytes in muscle, cardiomyocytes in heart, tubule cells in kidney, hepatocytes in liver) per grid were selected. The morphology of mitochondria was descriptively evaluated, and the number of mitochondria was manually counted in four blindly assessed fields of vision per cell at a magnification of 30,000 and represented as the number of mitochondria per 40 µm². Statistical analysis was performed with Student’s t test.

Mitochondrial/nuclear DNA ratio

Determination of the ratio between mitochondrial and nuclear DNA contents was performed by quantitative real-time PCR, essentially as described by others (21, 22). For DNA isolation, whole tissue samples from each eight WT and nNOS−/− mice were powdered in liquid nitrogen and 25 mg aliquots were used for DNA extraction by means of the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For each DNA extract, the nuclear gene for ribosomal protein large p0 and the mitochondrial gene cytochrome c oxidase subunit I (CoxI) were quantified separately by real-time PCR using the iCycler (Bio-Rad, Munich, Germany). The PCR reactions were performed in triplicate for each gene. A typical 25 µl reaction mixture contained 12.5 µl HotStarTaq Master Mix (Qiagen), 0.3 µl of a 1:1000 dilution of SYBR Green I (Molecular Probes, Eugene, OR), 100 ng DNA, and 0.5 µmol of the specific primers: p0-US 5′-GCACCTTCCGCTTTCTGGAGGTGT-3′ and p0-DS 5′-TGACTTGGTTGCTTTGGCCGGATT-3′; CoxI-US 5′-TCTACTATTTCCGAGGCTTGAG-3′ and CoxI-DS 5′-CTACTGATgcTCCTGGAATG-3′. An initial denaturation/activation step (15 min 95°C) was followed by 40 cycles (30 s 95°C, 30 s 58°C, 45 s 72°C).
Determination of citrate synthase activity (EC 2.3.3.1)

Citrate synthase activity was assayed using a standard procedure at 30°C (23). Homogenate samples of respective tissues (40 µg) were evaluated. The decrease of acetyl CoA absorption was monitored at 412 nm using a Cary 1E spectral photometer (Varian). The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 5 mM MgCl₂, 0.5 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 0.2 mM acetyl CoA, and 1 mM oxaloacetic acid.

Determination of citric acid and pyruvate

Isolated liver mitochondria from WT and nNOS-KO mice were incubated in air saturated incubation medium (~1 mg mitochondrial protein per ml) at 30°C in the presence of 20 mM pyruvate and 20 mM malate. After 20 min, samples of 2.5 ml volume were withdrawn from the incubation mixture and centrifuged at 10,000 g for 10 min. The supernatant was collected and saved for the determination of citric acid concentration. Finally, the accumulated citric acid was determined according to a standard procedure (24). Pyruvate was determined according to a classical standard procedure.

Determination of the lipid content in the brain, heart, and liver

After preparation, tissues were weighed, excised, and homogenized with a dounce homogenizer in 1 ml of incubation medium used for the preparation of mitochondria of the corresponding tissue. For the extraction of lipids, 5 ml of chloroform:methanol (2:1) was added and shaken for 1 h at 37°C. Afterward, homogenates were centrifuged at 3000 g for 10 min and the lower lipid containing phase was collected. The solvent was evaporated, and the total lipids were determined by dry weight.

Determination of protein

The protein content of the samples was determined by the Pierce bicinchoninic acid protein assay (25) using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis was carried out with ANOVA test. Data are presented as mean ± SE. Statistical comparisons were performed using Student’s t test; P < 0.05 was taken as the indicator of significance.

RESULTS

Distribution of nNOS

We compared the expression of nNOS in different tissues of WT and nNOS-KO animals by Western blot analysis to evaluate the distribution of this isoenzyme over the organism. Therefore, homogenates of the brain, muscle, heart, kidney, and liver were applied for Western blot analysis using a primary antibody directed against nNOS. The loading of equal amounts of protein at each lane of the gel is indicated by identical optical densities of the β-actin bands (Fig. 1, bottom). In all investigated tissues of WT animals, nNOS was detected with particularly high
amounts in brain and muscle. Relatively low contents of nNOS protein were found in the heart, kidney, and liver (Fig. 1, top). We were not able to detect significant amounts of nNOS protein in the brain, muscle, heart, kidney, and liver of nNOS-KO animals (Fig. 1, middle).

**Contents of mitochondria in WT and nNOS-KO mice in different tissues**

To study the effect of the presence of nNOS on the content of mitochondria in different tissues of mice, we applied several approaches. First, the number of mitochondria was investigated in tissue-specific cell types by Electron microscopic analysis. These experiments revealed that nNOS-KO mice tend to show a lower density of mitochondria expressed as the number of mitochondria per 40 µm² in neurons of the brain cortex (32.42±2.54 in WT vs. 25.08±2.04 in nNOS-KO, \( P<0.05 \)) and in tubule cells of the kidney (59.17±3.52 in WT vs. 47.92±3.25 in nNOS-KO, \( P<0.05 \)), whereas no significant differences were found in the myocytes of the gastrocnemius muscle, cardiomyocytes, and hepatocytes (Fig. 2). In addition, no evident change in the morphology of mitochondria by knocking out the nNOS isoenzyme was observed. In all tissue samples studied, mitochondria were variable in size and shape. Some of them were globular (0.5 µm or more in diameter), whereas others were elongated threads (0.1 µm in diameter and several microns long). Most of them contained longitudinally oriented cristae. Mitochondria of hepatic parenchyma were characterized by a dense matrix, whereas in brain cortex and myocardium the mitochondrial matrix was more lucent (electron microscopic graphs in Fig. 2).

In a second series of experiments, we compared in tissue homogenates of nNOS-KO and WT mice further parameters that are expected to correspond to the mitochondrial quantity. In contrast to the Electron microscopy analysis, this investigation takes all cell types of the investigated tissues into account. In this context, we evaluated the ratio of mitochondrial and nuclear DNA in the tissues of nNOS-KO and WT mice (6 animals per tissue and mouse model) by real-time PCR as described in Materials and Methods. No significant difference was found comparing the ratios of mitochondrial and nuclear DNA in the brain, muscle, heart, kidney, and liver. Likewise, we determined the tissue content of cytochrome c in the brain, muscle, heart, kidney, and liver (6 animals per tissue and mouse model) by Western blot analysis. The amount of this protein should strongly correlate with the amount of mitochondria under physiological conditions. For correct comparison of band intensities by using the BioDoc Analyze-System from Biometra, each gel contained the cytochrome c bands of both nNOS-KO and WT mice from one tissue. No significant difference was found between the band intensities of the brain, muscle, heart, kidney, and liver of nNOS-KO and WT animals.

Additionally, we determined the activity and the amount of protein of citrate synthase in tissue homogenates of WT and nNOS-KO animals. This enzyme is commonly used as a marker enzyme for the mitochondrial matrix. Surprisingly, between two and three times as high activities of citrate synthase were determined in the brain, muscle, kidney, and liver for nNOS-KO in comparison to WT animals (Fig. 3A). The Western blot analysis revealed that the expression of this enzyme was in tendency higher in brain and muscle and significantly higher in heart, kidney, and liver in nNOS-KO in comparison to WT mice (Fig. 3B). Since similar ratios of mitochondrial and nuclear DNA as well as contents of cytochrome c were determined in the tissues of WT animals, this observation documents significantly higher citrate synthase activities specifically in mitochondria of nNOS-KO mice.
Activities of respiratory chain complexes in WT and nNOS-KO mice

In another series of experiments, we investigated whether, and to what extent, the expression of nNOS affects the activity of respiratory chain complexes. To this end, the activity of the NADH:cytochrome c oxidoreductase enzyme complex was determined in homogenates of the brain, muscle, heart, kidney, and liver of WT and nNOS-KO mice. In the brain, muscle, and heart, the activity of the NADH:cytochrome c oxidoreductase complex was lower in nNOS-KO than in WT animals (Fig. 4). In the kidney and liver, however, the opposite was found. Here, the activity of the NADH:cytochrome c oxidoreductase complex was somewhat higher in the homogenates of nNOS-KO animals. A general tendency of lower values in nNOS-KO animals was seen when the NADH:cytochrome c oxidoreductase complex activity was related to the citrate synthase activity of the corresponding organ.

The tissues of WT animals contained equal (muscle) or higher activities of the succinate cytochrome c oxidoreductase activity (Fig. 5). When related to the activity of the citrate synthase, significantly lower values were determined for the homogenates of all tissues of nNOS-KO animals in comparison to WT mice. This reveals that, in comparison to WT animals, nNOS-KO mice are characterized by an excess of citrate synthase in relation to respiratory chain activity.

Effects of nNOS on the lipid content in the brain, heart, and liver

Our finding that the presence of nNOS has a significant effect on the activity of the citrate synthase stimulated us to investigate the effect of the lack of nNOS on lipid metabolism. Higher citrate synthase activity found in all evaluated tissues of nNOS-KO in comparison to WT animals should result in higher mitochondrial citrate concentration. Mitochondrially formed citrate can be released into the cytosol. Thus, an increase in mitochondrial citrate concentration should cause elevation of cytosolic citrate concentration. Citrate is known as a key metabolite in cytosolic fatty acid synthesis. Increase in cytosolic citrate concentration stimulates acetyl-CoA formation by the ATP-dependent citrate lyase. Subsequently, increase in fatty acid concentration should stimulate the formation of lipids resulting in elevated tissue lipid content. Based on these known metabolic possibilities, we hypothesize that nNOS-dependent modulation of citrate synthase activity may regulate the content of lipids in organs. Among the tissues used for the analysis of mitochondrial content and the activity of respiratory chain enzyme complexes, only in liver is fatty acid synthesis possible. Therefore, we determined the lipid content in the liver of nNOS-KO and WT mice. Additionally, we analyzed the lipid content in the brain and heart of these mouse models representing tissues without fatty acid synthesis. A higher content of total lipids was found in the livers of nNOS-KO mice in comparison to WT animals. However, no difference in the lipid content could be determined in the brain and heart when nNOS-KO mice were compared with WT animals (Fig. 6).

To further support the hypothesis that endogenous NO is involved in the regulation of fatty acid metabolism, we compared the accumulation of citric acid in the incubation medium that originated from isolated liver mitochondria of nNOS-KO and WT mice. Citric acid production was stimulated by incubating the mitochondria in the presence of 20 mM pyruvate and 20 mM malate for 20 min at 30°C. Afterward, the citric acid accumulated in the incubation medium was determined. The liver mitochondria of nNOS-KO mice released significantly more citric acid
into the incubation medium than mitochondria of WT animals (9.27±1.38 vs. 2.43±0.54 nmol/min/mg mitochondrial protein, first line in Table 1). This higher rate of citric acid accumulation was paralleled by a higher rate of pyruvate consumption by liver mitochondria of nNOS-KO in comparison to WT mice (80.77±10.81 vs. 45.98±6.94 nmol/min/mg mitochondrial protein, second line in Table 1). However, the comparison of these data demonstrates that most of the formed citric acid remains within the mitochondria probably as intermediate of the citric acid cycle under this condition.

To provide a further piece of evidence for the regulatory effect of endogenous NO generated by nNOS, we investigated the expression of the fatty acid synthase by Western blot analysis in the livers of nNOS-KO and WT mice. The corresponding data are depicted in the third line of Table 1. In the livers of nNOS-KO animals, about three times more fatty acid synthase protein was found in comparison to WT mice (24,989±6248 vs. 7826±1288 band intensity in arbitrary units).

**DISCUSSION**

We found the neuronal variant of nitric oxide synthase isoenzymes, nNOS, to be expressed in the brain, muscle, and even to a lesser extent in the heart, kidney, and liver in WT mice (see Fig. 1). There it contributes, together with the endothelial (eNOS) and possibly with a mtNOS isoform, to the formation and distribution of NO. It has been recognized that mitochondria are specific targets of NO (26–29). Besides metabolic effects, endogenous NO has most recently been shown to stimulate the biogenesis of mitochondria (13). The conclusions of Nisoli’s group (13) were derived from the comparison of eNOS-KO and WT mice by quantifying mitochondrial DNA, subunit IV of the cytochrome oxidase complex, and cytochrome c content in the brain, heart, and liver. Our electron microscopic analysis also revealed the tendency of lower numbers of mitochondria in special cells of the tissues of nNOS-KO compared with WT mice. This suggests that NO produced either by constitutive nNOS or by eNOS can stimulate the biogenesis of mitochondria, at least in special cell types such as neurons of the brain cortex and tubule cells of the kidney. The lower mitochondrial density in cells of tissues of nNOS-KO animals may not be in contradiction with the energetic requirements of the cell, since NO-dependent restriction of mitochondrial ATP production due to the inhibition of cytochrome c oxidase could be diminished. However, we were not able to demonstrate differences in the ratio of mitochondrial and nuclear DNA as well as in the content of cytochrome c in tissue homogenates between nNOS-KO and WT mice. Thus, the effect of the presence of nNOS on mitochondrial biogenesis seems to be restricted to special cell types. This is in contrast to the effect of eNOS on mitochondrial biogenesis that has been demonstrated also in tissue homogenates (13). However, the results of this study reveal that further investigation is required to elucidate the effect of endogenous NO on special cell types in complex tissues such as brain.

Our data suggest that nNOS-derived NO causes increased tissue content of the succinate cytochrome c oxidoreductase measured in the brain, heart, kidney, and liver, and in the NADH:cytochrome c oxidoreductase activities measured in the brain, muscle, and heart of WT mice. Higher amounts of respiratory chain enzyme complexes may compensate for the decrease in mitochondrial ATP production due to the inhibition of cytochrome c oxidase by NO, demonstrated many times in several laboratories (30–33). These differences in activities of respiratory chain enzyme complexes did not correlate with the tissue contents of cytochrome c of nNOS-KO and WT animals, although cytochrome c is involved in the electron transport within
the respiratory chain. Since only that part of the cytochrome $c$ pool that is tightly bound to mitochondrial inner membrane transfers electrons to complex IV of the respiratory chain (34), nNOS-dependent changes must not necessarily be reflected by changes of the whole cytochrome $c$ pool.

An essential result of our study is the observation that mitochondria of nNOS-KO mice exert up to three times the levels of citrate synthase protein and activities compared with mitochondria of WT animals. This is surprising since these animals had similar amounts of mitochondria in the tissues homogenates that had been investigated. The nNOS-dependent changes in the distribution of mitochondrial enzyme activities were most evident when the activities-related data, e.g., ratios of the activities of mitochondrial enzymes, were calculated. Clear differences in the relationship between respiratory chain enzyme complex activity and citrate synthase activity in tissues of WT and nNOS-KO mice became evident when the respiratory chain complex activities were related to the corresponding citrate synthase activity. Specifically increased levels of citrate synthase activity and protein were recorded here for the first time for the mitochondria of mice deficient in nNOS. Our data suggest that the higher activities of citrate synthase in the tissues of nNOS-KO mice are, at least partially, based on higher amounts of the protein due to stimulation of expression. As described in Materials and Methods, the activity of the citrate synthase was determined in tissue homogenates ruling out the direct influence of reactive oxygen species and NO, which require functional intact cells. Thus, it is reasonable that the presence of nNOS suppresses the expression of citrate synthase in the investigated tissues. Effects of nNOS on citrate synthase may be restricted on specific cell types or require a long period of time since in a study with cultured endothelial cells that were exposed to NO over 24 h no effect of NO was found on the activity of citrate synthase (35).

It has been reported that NO inhibits the aconitase reaction, which consumes citrate as substrate (36). From this it might be argued that because of increased aconitase activity, the high activity of the citrate synthase found in tissues of nNOS-KO mice must not necessarily result in higher mitochondrial citrate concentrations in comparison to WT animals. However, we have found that the respiratory chain enzyme complex activities are rather lower in nNOS-KO in comparison to WT mice. Therefore, a higher turnover rate of the citric acid cycle is unlikely, and consequently, the concentration of citrate and other intermediates of the citric acid cycle should increase. A rise in the citrate concentration in the mitochondrial matrix may result in an increase in the cytosolic citrate concentration via transport through the mitochondrial membrane. This hypothesis is strongly supported by our finding of higher extramitochondrial concentration of citric acid originated from functional intact liver mitochondria of nNOS-KO in comparison to WT mice. Increase in cytosolic citric acid concentration may cause stimulation of acetyl-CoA formation by the ATP-dependent citrate lyase. Since citrate lyase is known to be a key enzyme in fatty acid metabolism, the elevation of the cytosolic citrate concentration favors the formation of fatty acid. Moreover, we have found higher amounts of fatty acid synthase protein in nNOS-KO in comparison to WT mice. This enzyme might be a further target of nNOS-dependent regulation of fatty acid synthesis in liver. Thus, a previously unrecognized effect of NO appears to be the regulation of fatty acid synthesis by suppressing the rate of citrate synthesis in mitochondria and the expression of fatty acid synthase. Recently, an additional influence of NO on fatty acid synthesis was reported. It was shown that NO can nitrosylate acetyl CoA to the metabolically inactive S-nitrosoCoA (37), resulting in a direct and short-term control of fatty acid synthesis.
Effects of NO on lipid metabolism should be immediately reflected in the tissue content of lipids and ultimately in NO-dependent body weight changes. In fact, the latter was demonstrated by Nisoli et al. (13) showing higher body weights of eNOS-KO mice in comparison to WT animals. These differences were concluded to be the result of the NO-dependent stimulation of mitochondrial biogenesis. We did not find any difference in the body weights of WT and nNOS-KO mice in our study (data not shown). In contrast to eNOS-KO mice, nNOS-KO are characterized by a higher locomotive activity compared with WT animals. This may explain our unsuccessful effort in detecting differences in the body weight of WT and nNOS-KO animals. However, we found higher contents of lipids in the livers, but not in the brains and hearts, of nNOS-KO mice in comparison to WT animals. Among the tissues investigated only liver is equipped with the enzymes required for fatty acid synthesis. Therefore, our finding of higher lipid content in the liver of nNOS-KO mice further supports the suggestion of higher mitochondrial and cytosolic citrate concentration. Moreover, these data support the hypothesis that NO exerts a regulatory function in lipid metabolism at the level of citrate synthase and fatty acid synthase. This NO-dependent regulation of fatty acid synthesis may, in addition to the control of mitochondrial densities by NO, contribute to differences in body weight between WT and eNOS-KO mice as were found by Nisoli et al. (13).

Here, we report for the first time that NO derived from constitutive nNOS plays a crucial role in the activity pattern of mitochondrial enzymes. In particular, the NO-mediated suppression of citrate synthase activity and protein, reported here, may be attributed to a regulatory function of NO in fatty acid synthesis. Inhibition of mitochondrial respiration by NO appears to be, at least partially, compensated for by a respective increase in the respiratory chain enzyme complexes.

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REFERENCES


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### Table 1

**Effect of the expression of the nNOS isoenzyme on extramitochondrial citric acid accumulation and expression of fatty acid synthase protein**

<table>
<thead>
<tr>
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<th>WT</th>
<th>nNOS-KO</th>
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<tr>
<td><strong>Citric acid accumulation</strong></td>
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<tr>
<td>(nmol/min/mg mitochondrial protein)</td>
<td>2.43 ± 0.54</td>
<td>9.27 ± 1.38*</td>
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<tr>
<td><strong>Pyruvate consumption</strong></td>
<td></td>
<td></td>
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<tr>
<td>(nmol/min/mg mitochondrial protein)</td>
<td>45.98 ± 6.94</td>
<td>80.77 ± 10.81*</td>
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<tr>
<td><strong>Fatty acid synthase protein</strong></td>
<td></td>
<td></td>
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<tr>
<td>(band intensity in arbitrary units)</td>
<td>7826 ± 1288</td>
<td>24,989 ± 6248*</td>
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Accumulation of citric acid mitochondria in incubation medium generated by isolated liver mitochondria is shown. The respective pyruvate consumption and amount of fatty acid synthase protein (determined in liver homogenates by Western blot analysis) of nNOS-KO and respective WT mice are presented. For determination of citric acid concentration and pyruvate consumption, isolated liver mitochondria were incubated for 20 min in the presence of 20 mM pyruvate and 20 mM malate. Mean values ± SE from 6 animals of each group are shown. *Differences between data of nNOS-KO and WT mice are significant with $P < 0.05$, according to Student’s *t* test.
Figure 1. Distribution of nNOS protein. Western blot analysis in the respective homogenates of WT mice demonstrates nNOS expression, although with different intensity, in all tissues studied, whereas samples of nNOS-KO mice are free of detectable amounts of nNOS protein. Shown is 1 representative experiment out of 5 preparations. Protein amount per lane was 20 µg. β-Actin bands demonstrate equal sample loading. Purified nNOS protein distributed by BD Biosciences was used as control (first lane).
Figure 2. Effect of nNOS-derived NO on number of mitochondria. Electron microscopic evaluation demonstrates a significantly reduced density of mitochondria (data are expressed in number of mitochondria per 40 µm²) in cortical neurons of brain and in distal tubule cells of kidney of nNOS-KO mice (*<0.05). This tendency was also found in myocytes of gastrocnemius muscle and in cardiomyocytes but not in hepatocytes. Morphology of mitochondria was independent of endogenous NO supply. Without exception, the organ-specific pattern of well-defined cristae structures is seen.
Figure 3. Effect of nNOS-derived NO on activities of mitochondrial citrate synthase (A) and amount of citrate synthase protein (B). Citrate synthase activities were determined in homogenates from tissues of nNOS-KO and respective WT mice. Activities of citrate synthase are given in µmol/min/mg protein of homogenate. Data are mean values ± SE of 10 preparations. Amount of citrate synthase protein was analyzed in tissue homogenates of nNOS-KO and respective WT mice by Western blot technique. Protein amount per lane was 20 µg. β-Actin bands demonstrate equal sample loading. Band intensities were quantified by using BioDoc Analyze-System (Biometra) and given as mean ± SE in arbitrary units from 5 preparations. *Difference between data of a tissue from WT and nNOS-KO mice was significant with $P < 0.05$. 

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT (µmol/min/mg) ± SE</th>
<th>KO (µmol/min/mg) ± SE</th>
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<tr>
<td>Brain</td>
<td>29.367 ± 3.590</td>
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<td>Muscle</td>
<td>19.080 ± 1.380</td>
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<tr>
<td>Heart</td>
<td>14.517 ± 2.475</td>
<td>37.659 ± 8.668</td>
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<tr>
<td>Kidney</td>
<td>70.380 ± 8.490</td>
<td>119.110 ± 20.845</td>
</tr>
<tr>
<td>Liver</td>
<td>52.832 ± 6.620</td>
<td>81.184 ± 7.641</td>
</tr>
</tbody>
</table>

Beta-Actin bands demonstrate equal sample loading.
Figure 4. Effect of nNOS-derived NO on the activity of the NADH:cytochrome c oxidoreductase complex. NADH:cytochrome c oxidoreductase activities were determined in homogenates from tissues of nNOS-KO and respective WT mice. Activities are related on mg protein of homogenate. All data are mean values ± SE of 10 preparations. *Difference between the activities of tissue from WT and nNOS-KO mice was significant with $P < 0.05$. 
Figure 5. Effect of nNOS-derived NO on activity of the succinate (Succ) cytochrome c oxidoreductase complex. Succinate cytochrome c oxidoreductase activities were determined in homogenates from tissues of nNOS-KO and respective WT mice. Activities are related on mg protein of homogenate. All data are mean values ± SE of 10 preparations. *Difference between activities of tissue from WT and nNOS-KO mice was significant with $P < 0.05$. 
Figure 6. Effect of expression of the nNOS isoenzyme on tissue lipids. Total lipid content determined for brain, heart, and liver of nNOS-KO and respective WT mice is presented as ratio of lipid and tissue weight as mean ± SE of at least 6 preparations of tissue homogenates. *Difference between activities of tissue from WT and nNOS-KO mice was significant with $P < 0.05$. 

**Figure 6.** Effect of expression of the nNOS isoenzyme on tissue lipids. Total lipid content determined for brain, heart, and liver of nNOS-KO and respective WT mice is presented as ratio of lipid and tissue weight as mean ± SE of at least 6 preparations of tissue homogenates. *Difference between activities of tissue from WT and nNOS-KO mice was significant with $P < 0.05$. 

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