Autoregulation of 1,25-Dihydroxyvitamin D Synthesis in Macrophage Mitochondria by Nitric Oxide

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Tissue macrophages from patients with granuloma-forming diseases, most notably sarcoidosis, express a 25-hydroxyvitamin D-1-hydroxylase which can produce in vivo sufficient quantities of the active vitamin D metabolite 1,25-dihydroxyvitamin D to cause hypercalcemia. In contrast to the NADPH-dependent cytochrome P450-linked mixed function oxidase which is normally only expressed in significant quantity in proximal renal tubular cells and regulated in an endocrine fashion, the mitochondrial-based 1-hydroxylase in the macrophage [1] is stimulated in a paracrine mode by cytokines (i.e., IFN-gamma) and lipopolysaccharide (LPS) [2] requires an extracellular source of L-arginine for full basal expression and [3] can be regulated in an intracrine fashion by nitric oxide (NO). In these experiments we employed inducible nitric oxide synthase (iNOS)-free, intact mitochondria preparations from the avian macrophage-like cell line HD-11, which constitutively express the 1-hydroxylase, and nonenzymatically-generated NO to investigate NO-mediated autoregulation of the macrophage 1-hydroxylase. Sodium nitroprusside (SNP)- or S-nitroso-N-acetyl-penicillamine (SNAP)-induced up-regulation of the 1-hydroxylase required the presence of either NADPH or NADP in the reaction mixture, while NO-induced inhibition of mitochondrial 1,25-(OH)2D3 synthesis was NO-dependent and NADP/NADPH-independent, indicating different regulatory mechanisms for basal and NO-mediated expression. The NO-mediated regulation of the 1-hydroxylase is biphasic. At relatively high concentrations NO competes with O2 for enzyme-bound iron, inhibiting enzyme synthesis. At lower production levels, NO serves as a source of reducing equivalents for the enzyme by providing for the reduction of NADP to NADPH.

1,25-Dihydroxyvitamin D (1,25-(OH)2D) is the hormonal form of vitamin D. As such, circulating concentrations of the hormone are stringently regulated to maintain normal calcium homeostasis. Control of 1,25-(OH)2D hormone levels occurs primarily at the level of hormone synthesis (1); in normal mammals and birds the enzyme responsible for hormone production is the 25-OHD-1-hydroxylase (2). The 1-hydroxylase is located principally in proximal renal tubular epithelial cells (3). The activity of the 1-hydroxylase is regulated primarily in an endocrine fashion by the circulating level of parathyroid hormone (PTH) (4). The so-called renal 1-hydroxylase has been functionally categorized as a cytochrome P450-linked oxioreductase dependent upon NADPH as a source of electrons, a reductase and a ferredoxin as electron donating accessory proteins, and molecular oxygen (2). The renal 1-hydroxylase is a mitochondrial enzyme (5,6).

The kidney is not the sole human source of enzyme with 25-OH-1-hydroxylating potential. Disease-activated tissue lymphoma (8,9), also harbor a 25-OHD3-1-hydroxylase. The macrophage 1-hydroxylase is induced in an intracrine fashion by nitric oxide (NO). In these experiments we employed inducible nitric oxide synthase (iNOS)-free, intact mitochondria preparations from the avian macrophage-like cell line HD-11, which constitutively express the 1-hydroxylase, and nonenzymatically-generated NO to investigate NO-mediated autoregulation of the macrophage 1-hydroxylase. Sodium nitroprusside (SNP)- or S-nitroso-N-acetyl-penicillamine (SNAP)-induced up-regulation of the 1-hydroxylase, and nonenzymatically-generated NO to investigate NO-mediated autoregulation of the macrophage 1-hydroxylase. At relatively high concentrations NO competes with O2 for enzyme binding, inhibiting hormone synthesis. At lower production levels, NO serves as a source of reducing equivalents for the enzyme by providing for the reduction of NADP to NADPH.

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Materials: Crystalline 25-OH3D and 1,25-(OH)2D3 were kindly provided by M. Uskokovic (Hoffmann-LaRoche, Nutley, NJ) [6,27, methyl-3H]25-OH2D3 (specific activity 181 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Organic solvents for vitamin D extraction were all high performance liquid chromatography grade from Baxter (Muskegon, MI). C18 and silica Sep-Pak cartridges employed for lipid extraction and chromatography were purchased from Millipore (Milford, MA). Cell culture medium supplements were from Gibco (Gaithersburg, MD). Sodium nitroprusside (SNP) and all other effector substances and buffer components were from Sigma (St. Louis, MO). S-nitroso-N-acetylpenicillamine (SNAP) was synthesized for use as previously described (10). Vitamin D receptor was purchased from Incstar (Stillwater, MN).

Cell Culture and Subcellular Fractionation Procedures: The HD-11 macrophage cell line (17) was grown in monolayers in MEM supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 u/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere of 5% CO2 and 95% air. When confluent, the medium was aspirated and the cells washed twice with indicator-free Earle's Balanced Salt Solution (EBSS). The monolayers were then incubated for 24 h in FBS-free, phenol red-free MEM supplemented with L-glutamine and antibiotics. The cells were harvested with 1% trypsin-EDTA (Thermoscientific, CA) at 37°C. After neutralization of the trypsin, cells were washed twice with ice-cold TMSS buffer (10 mM Tris-base, 2 mM MgCl2, 2.5 mM sucrose and 25 mM sucrose, pH 7.4). The washed cells (2x10^7) were resuspended in TMSS buffer, disrupted by sonication (W-300, Ultrasonic, Farmingdale, NY), and sonicated for 10 min at 4°C to remove nuclei and plasma membrane debris, at 8,500xg for 10 min at 40°C to recover mitochondria, and finally at 100,000xg for 1 h at 4°C to remove microsomes from the cytosolic fraction. The mitochondria were frozen in TMSS buffer and stored at -70°C until use. The functional integrity of the mitochondria was confirmed by their ability to oxidize reduced cytochrome C substrate (10). The cytosol fraction was concentrated 40-fold (volume:volume) by filtration through a 10K YM Amicon membrane (10
Measurement of Mitochondrial Nitrite Production: Triplicate aliquots (100 μl) of suspended mitochondria were incubated in TMS buffer (10 mM phosphate buffer at pH 7.4) in the presence or absence of effector agents for 3 h at 37°C. The reaction was collected and cleared of cells by centrifugation. Duplicate aliquots of supernatant from each reaction were employed for measurement of the stable water soluble metabolite of NO, nitrite, via the Griess reaction in 96-well microtitrato (Costar) (19).

Measurement of 1,25-(OH)2D3 Synthesis by Isolated Mitochondria: Triplicate aliquots (100 μl) of intact HD-11 cell mitochondria were incubated in TMS with 100 μM substrate 25-OHD3 and with or without effector agents for 3 h at 37°C. The incubation was terminated by the addition of an equal volume (1 ml) acetonitrile to extract 25-OHD3 metabolites from the reaction mix containing conditioned supernatant and mitochondrial pellet. Quantification of 1,25-(OH)2D3 produced was determined by V87-radiogand binding assay of Reinhardt (20). Recovery of product 1,25-(OH)2D3 through lipid extraction and chromatography was monitored by the addition of 5 fmol [3H]1,25-(OH)2D3 to each sample. All samples from a single experiment were measured in the same assay; the intra-assay coefficient of variation was 8.4%. When samples from multiple experiments were employed to assess the potential regulatory influence of a specific factor or condition on metabolite production, results were expressed in terms of basal expression of the 1-hydroxylating reaction.

Statistical Analyses: Where appropriate, Student’s t-test was used in analysis of intergroup statistical differences.

RESULTS AND DISCUSSION

Tissue macrophages harvested from patients with active granuloma-forming diseases constitutively express a 25-OHD-1-hydroxylating reaction (21). The avian myelomonocytic cell line HD-11 expresses a 1-hydroxylating reaction that is similar in all respects to that found in primary cultures of human macrophages (12), so synthesis of 1,25-(OH)2D3 from 25-OHD3 in this cell line has been employed by us as a model of the human macrophage 1-hydroxylase. Because the 1-hydroxylase in macrophage-like cells of either human or avian origin is stimulated by interferon-gamma (IFN) and lipopolysaccharide (LPS) (18,12), recognized stimulators of the iNOS in macrophages (22), we queried whether NO might be important in basal expression and in upregulation of the macrophage 1-hydroxylase. We previously showed that an extracellular source of L-arginine and functional NOS were required for the basal 1,25-(OH)2D3 synthetic capacity of intact HD-11 cells (14). More recently (15) we showed that increasing NO production in HD-11 cells, either endogenously with LPS or exogenously by SNP addition, was associated with an initial increase in 1,25-(OH)2D3 production followed by a downturn in hormone synthesis as cellular NO production continued to increase. We have proposed that this biphasic modulation of HD-11 cell 1,25-(OH)2D3 production is coordinated physicochemically by NO; relatively low intracellular concentrations of NO act to increase 1,25-(OH)2D3 synthesis by serving as an electron donor for the 25-OHD-1-hydroxylase, while at higher intracellular levels NO begins to compete with O2 for binding to this oxidase. If our theory is correct, then non-enzymatic modulation of NO production in vitro should result in autoregulation of 1,25-(OH)2D3 synthesis in whole mitochondria bearing the 1-hydroxylase.

Figure 1 shows the functional integrity of basal cytochrome C oxidizing and 1-hydroxylating activity in mitochondria isolated from confluent HD-11 cell cultures. Panel a shows the expected rapid decline in reduced cytochrome C in HD-11 cell mitochondrial preparations with oxidative potential; two have previously demonstrated similar activity in preparations of intact HD-11 cells (14). Panel b shows the 1,25-(OH)2D3 synthetic capacity of HD-11 cell mitochondria in the presence and absence of potential cofactors for the enzyme. As predicted, hormone production was not observed in the absence of enzyme (mitochondria) or substrate. Mitochondria were also incapable of hormone production in the absence of an electron source; addition of 1 mM NADPH resulted in a significant 5-fold rise in 1,25-(OH)2D3 synthesis. In the absence of NADPH addition of a 10x concentrated cytosol fraction to the reaction mixture was not sufficient for expression of the reaction and was not additive to NADPH in promoting 1-hydroxylating activity, indicating that isolated macrophage mitochondria required only an electron source for reconstituted expression.

Figure 1. Functional integrity of cytochrome-associated enzymes and the nonenzymatic NO generating capacity of isolated mitochondria from HD-11 cells. Panel a shows the decrease (oxidation) in reduced cytochrome C in HD-11 cell mitochondria with oxidative potential. Each point is the mean of duplicate observations made at least once a minute for eight minutes. Panel b shows an increase in 1,25-(OH)2D3 synthesis by HD-11 cell mitochondria only in the presence of NADPH and substrate. Data (Mean±SD) for each condition were derived from two different experiments each performed in triplicate; the mean basal 1,25-(OH)2D3 level was 120 pg/assay tube. Panel c depicts NO (nitrite) generating capacity of intact mitochondria (mito) before and after incubation with the NO donor sodium nitroprusside (SNP, 200 μM) in the presence or absence of the cofactor NADPH (1 mM). As anticipated, an increase in NO production required mitochondria and SNP but was not altered by NADPH. Data are expressed as the mean±SD.
Figure 2. SNP concentration-dependent change in NO and 1,25-(OH)2D synthesis by HD-11 cell mitochondria. Panel a shows a significant SNP-dependent increase in NO (nitrite) accumulation, while panel b describes a significant, uniphasic decrease in vitamin D hormone synthesis coincident with the SNP-induced increase in NO. Panel c shows restoration of a significant, biphasic, SNP-mediated change in mitochondrial 1,25-(OH)2D production in the presence of 1 mM NADPH. All data are expressed as the mean±SD of basal expression in at least two different experiments; the mean basal 1,25-(OH)2D3 level among all experiments was 98 pg/assay tube. Panel d is our working model for biphasic effect of NO on the mitochondrial 1-hydroxylase in mitochondria. The stimulatory effect on the enzyme is mediated by relatively low intracellular NO levels. An electron (e-) generated from NO is donated to oxidized NADP+ to form NADPH. NADPH, in turn, supplies the electron transport chain of accessory proteins, consisting of a flavoprotein reductase (FP), a ferredoxin (Fdx), and a cytochrome P450, linked to the 1-hydroxylase. On the other hand, the inhibitory effect of relatively high NO levels in the cell results from competition with O2 binding to the P450 heme group.

Although iNOS is believed to be responsible for the bulk of NO produced by macrophage-like cells (23), in the absence of iNOS NO can be generated nonenzymatically from sodium nitroprusside (SNP). Figure 1c shows the NO generating capacity of HD-11 cell mitochondria after exposure to 200 μM SNP. In the absence of SNP, HD-11 cell mitochondria produced barely detectable amounts of nitrite, not different from that obtained in buffer alone. These results support the assumption that these mitochondrial preparations were devoid of endogenous iNOS activity. Addition of 200 μM SNP stimulated an increase in nitrite production and NADPH was not required for the SNP-induced NO synthesis.

We have theorized (15) that NO, a small lipid-soluble molecule containing an unpaired electron in its outer shell (24), might be an ideal electron donating candidate for electron-requiring P450 enzymes, like the 25-OHD-1-hydroxylase, that are embedded in the membranes of intracellular organelles. Considering that [1] HD-11 cell mitochondria can synthesize 1,25-(OH)2D3 when supplied in vitro with substrate 25-OHD3 and a source of electrons and [2] NO can be generated from SNP in vitro by mitochondria, we next asked the question whether NO could substitute for NADPH in support of the mitochondrial 1-hydroxylase. From previous work with intact HD-11 cells (15), we estimated that a nitrite accumulation rate in the range of 2-3 mmol/ml/h (over a 3-h incubation period) would be required to promote the 1-hydroxylase in mitochondria and that rates >4 mmol/ml/h would result in inhibition of hormone synthesis; it is clear (Figure 1c) that NO production rates in this range can be achieved with mitochondria incubated with SNP. Panel a, Figure 2 shows the SNP concentration-dependent increase in nitrite accumulation in HD-11 cell mitochondria. Incubation of mitochondria with as little as 0.5 μM SNP resulted in a significant increase in NO. The ED50 for SNP-induced NO production was approximately 40 μM.

These same mitochondrial preparations were also employed to determine 1,25-(OH)2D3 production from 25-OHD3 (panel b, Figure 2). In contrast to the biphasic effect of NO on 1,25-(OH)2D3 production we previously observed in intact HD-11 cells (15), incubation of intact mitochondria with increasing amounts of SNP led only to a dose-dependent decrease in 1,25-(OH)2D3 production. The ED50 for SNP-mediated inhibition of vitamin D hormone production was 4 μM, indicating that inhibition of the 1-hydroxylase was an order of magnitude more sensitive to SNP than was detectable...
nitrate formation in mitochondria. Addition of 1mM NADPH to the SNP-treated mitochondria (panel c, Figure 2) restored the biphasic effect of NO on the 1-hydroxylase. In other words, a concentration of SNP (0.5μM) which was inhibitory in the absence of added NADPH now elicited a significant increase in hormone production. However, despite the presence of NADPH, exposure to higher concentrations of SNP (i.e., 50μM) still resulted in a downturn in 1,25-(OH)2D3 synthesis. To confirm that NO and not some other product of SNP was the electron donor in the reaction, we also tested the ability of SNAP, a synthetic compound which serves as a relatively pure NO donor (16), to stimulate the 1-hydroxylase; incubation with a concentration of SNAP (20μM) that induced a significant increase in nitrite accumulation (p<0.001) also significantly increased 1,25-(OH)2D3 synthesis (p<0.005).

The schematic in panel d, Figure 2 depicts the proposed transfer of electrons from NADPH to the 25-OHD-1-hydroxylase in our preparations of intact HD-11 cell mitochondria. In this model, an electron is donated by an extramitochondrial source of NADPH in serial fashion to three accessory proteins, to a flavin domain of a reductase, then to the non-heme iron protein (ferredoxin), and finally to the heme iron of the cytochrome-linked 1-hydroxylase. When O2 is present the enzyme catalyzes the hydroxylation of 25-OHD to 1,25-(OH)2D. We postulate that the inhibitory arm of the biphasic effect of NO on the mitochondrial 1-hydroxylase results from direct interference of NO in the oxidative reaction. It has recently been reported that NO, like carbon monoxide, can competitively inhibit O2 binding to P450 linked proteins, including NOE itself (25).

Unlike our previous observations with intact HD-11 cells (15), SNP-generated NO was found to be not sufficient for stimulation of the 1-hydroxylase in isolated mitochondria unless either NADP or NADPH was present in the reaction mixture (Figure 2). Based on these data we theorized that extramitochondrial NADP will accept an electron from NO and then a the non-heme iron protein (terredoxin), and finally to the heme iron of the cytochrome-linked 1-hydroxylase. When O2 is present the enzyme catalyzes the hydroxylation of 1,25-OHD to 1,25-(OH)2D. We postulate that the inhibitory arm of the biphasic effect of NO on the mitochondrial 1-hydroxylase results from direct interference of NO in the oxidative reaction. It has recently been reported that NO, like carbon monoxide, can competitively inhibit O2 binding to P450 linked proteins, including NOE itself (25).

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