

# Oxidation of $N^G$ -hydroxyl-L-arginine to nitric oxide mediated by respiratory burst: an alternative pathway to NO synthesis

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**Abstract**  $N^G$ -Hydroxy-L-arginine is an intermediate metabolite in the synthesis of nitric oxide that is upregulated and secreted during acute inflammation *in vivo*. Previous reports have shown that chemically induced superoxide anion oxidizes  $N^G$ -hydroxy-L-arginine to nitric oxide. Here, we demonstrate that this reaction takes place physiologically in phagocytic cells during the respiratory burst, and is independent of the presence of nitric oxide synthase.

**Key words:** Nitric oxide; Superoxide anion; Hydroxy-L-arginine; Respiratory burst; Phagocyte

## 1. Introduction

Nitric oxide (NO) is a multifunctional compound implicated in a variety of physiological functions including the cytotoxic effector phase of macrophages [1]. NO is produced in cells and tissues by several different types of nitric oxide synthases (NOS). All of these enzymes share the same two step mechanism in NO synthesis: (i) the oxidation of arginine to  $N^G$ -hydroxy-L-arginine (OH-Arg), and (ii) the oxidation of OH-Arg to NO and citrulline [2]. While the participation of NOS is essential in the first step, the second step may be carried out by other oxidases including P450 [3]. Recently, Sennequier et al. have shown that superoxide anion ( $O_2^-$ ) can induce the formation of nitrogen oxides by oxidation of  $N^G$ -hydroxyguanidines [4]. Furthermore, Everett et al. have shown that radiation induced  $O_2^-$  mediates denitrification of OH-Arg leading to NO and cyclized carbodiimide [5]. A biological role of this alternative pathway of NO synthesis is suggested by the fact that the intermediate product of enzymatic nitric oxide synthesis, OH-Arg, is elevated in sera of animals treated with LPS [6] and is secreted together with NO *in vivo* and *in vitro*, as an adduct [7]. Here we demonstrate in cell culture that the superoxide anion produced by phagocytic cells during the respiratory burst is sufficient to induce NO from OH-Arg, and that this reaction is independent of the presence of NOS.

## 2. Materials and methods

$N^G$ -Hydroxy-L-arginine was obtained from Calbiochem (Bad Soden, Germany), lucigenin and superoxide dismutase (SOD), xanthine oxidase (Boehringer Mannheim, Germany) xanthine, PMA and zymosan (Sigma).

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**Abbreviations:** OH-Arg,  $N^G$ -hydroxy-L-arginine; BMMΦ, bone marrow-derived macrophages;  $O_2^-$ , superoxide anion.

### 2.1. Cells

Murine bone marrow-derived macrophages (BMMΦ). Bone marrow cells were obtained by flushing the femurs from CBF1 or iNOS knock out C57/Bl6×129Sv mice [8] and were cultured as previously described [9] for 11 days in hydrophobic Teflon bags (Biofolie 25, Heraeus, Hanau, Germany) in Dulbecco's modified Eagle's medium (DMEM) containing 10% inactivated foetal calf serum, 5% horse serum, 1 mM sodium pyruvate, 2 mM glutamine, 60 μM β-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Paisley, UK) and the supernatant of L929 fibroblasts at a final concentration of 15% (v/v) as a source of colony stimulating factors which drive cell proliferation towards a greater than 95% pure population of BMMΦ. Human granulocytes and monocytes were obtained from fresh human peripheral blood as described [10]. Leukocyte suspensions were prepared after sedimentation of erythrocytes in 1.3% Dextran (Pharmacia) at room temperature. The leukocyte-rich supernatant was centrifuged at 400×g for 40 min and the pellet was gently resuspended in 20 ml phosphate-buffered saline, loaded onto a layer of 20 ml Ficoll-Paque (Pharmacia), and centrifuged at 400×g for 40 min at room temperature to separate mononuclear cells from granulocytes. Contaminating erythrocytes in the granulocyte-rich pellet were eliminated by hypotonic shock and the remaining cells washed twice with phosphate-buffered saline. The final preparation contained more than 98% granulocytes, as assessed by Giemsa-Wright stain. The mononuclear cells from the PBS/Ficoll interface were washed three times with PBS, resuspended in DMEM+10% FCS and cultured in plastic Petri dishes for 2 h at 37°C. Non-adherent lymphocytes were carefully resuspended and discarded. The monocyte-rich adherent cells were scraped, washed once and resuspended in HEPES-DMEM.

### 2.2. Synthesis and determination of superoxide anion

Superoxide anion ( $O_2^-$ ) was produced by the xanthine/xanthine oxidase reaction. For this purpose xanthine was dissolved in HEPES buffered DMEM (without bicarbonate, indicator and arginine), pH 7.5 at a final concentration of 10 mM. The reaction was triggered by addition of 10 mU/ml xanthine oxidase (10 μl).  $O_2^-$  was measured as lucigenin-dependent chemiluminescence using a Microlumat LB96P (Berthold, Wildbad, Germany) [11]. 0.1 ml of the xanthine solution were placed into the microtiter plate and lucigenin (125 μM) and xanthine oxidase were added immediately before the measurement. The final volume was 0.2 ml. The  $O_2^-$  production by phagocytes ( $2 \times 10^5$  cells in 0.2 ml HEPES-DMEM and lucigenin) was initiated by addition of 250 μg/ml zymosan or 10 ng/ml PMA. Phagocytes ( $10^6$ /ml) were cultured in HEPES DMEM supplemented with 0.1 mM OH-Arg for 2 h as indicated in the figures.  $O_2^-$  was decomposed by addition of 100 U/ml of SOD. All enzymatic and cellular experiments were performed at 37°C. Results of the experiments are the mean values ± S.D. of quadruplicate experiments.

### 2.3. Determination of NO

Cell supernatants or xanthine/xanthine oxidase solutions were assayed for the stable end product of NO synthesis,  $NO_2^-$  using the Griess reagent. Briefly, cell-free supernatants (0.1 ml) were mixed with 100 μl of 1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5%  $H_3PO_4$ . Absorbance was measured at 540 nm in a ELISA reader. Results of all experiments are expressed as mean values ± S.D. of quadruplicate cultures.

### 3. Results

#### 3.1. Induction of nitrites by enzymatically induced $O_2^-$

In a first set of experiments, we determined the induction of  $O_2^-$  by the xanthine/xanthine oxidase system in the presence or absence of OH-Arg. Although optimal  $O_2^-$  synthesis takes place at pH levels above 8.0, a considerable amount of radicals is also induced at physiological pH, as shown in Fig. 1A. Furthermore, OH-Arg partially inhibits lucigenin-dependent chemiluminescence induced by  $O_2^-$ , presumably due to the consumption of  $O_2^-$  radicals for oxidation of OH-Arg. In contrast, induction of  $O_2^-$  was totally inhibited by SOD. None of the other reagent combinations gave rise to the induction of  $O_2^-$  (Fig. 1A). In a parallel experiment without lucigenin, the time course of the formation of nitrites was analyzed. The results in Fig. 1B demonstrate that NO synthesis is induced only in the presence of OH-Arg and when the production of the  $O_2^-$  was ensured by the xanthine/xanthine

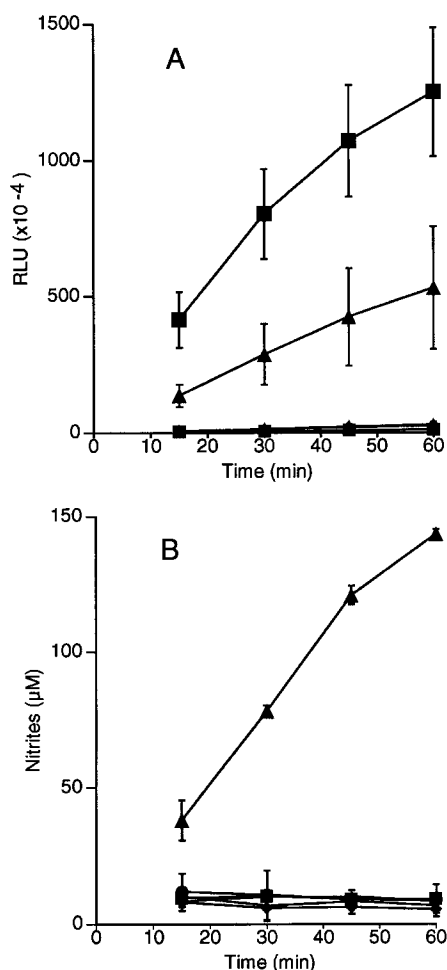


Fig. 1. Induction of NO by the  $O_2^-$  mediated oxidation of OH-Arg. (A) Superoxide anion induced luminescence was measured as described in Section 2. Xanthine (□), xanthine+xanthine oxidase (■), xanthine+xanthine oxidase+100 U/ml SOD (△), xanthine+1 mM OH-Arg (●), xanthine+xanthine oxidase+1 mM OH-Arg (▲), xanthine+xanthine oxidase+1 mM OH-Arg+100 U/ml SOD (◆). The data represent the integration of the induced cpm at the indicated times. (B) Parallel experiments without lucigenin in a volume of 1 ml were performed and at the indicated times the nitrite concentration was determined in aliquots of 0.1 ml. Symbols as in (A).

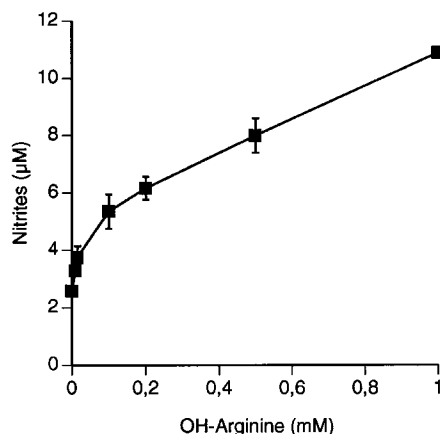


Fig. 2. Dependence of NO production mediated by the respiratory burst upon the OH-Arg concentration. BMMΦ were cultured for 2 h in the presence of the indicated amounts of OH-Arg and zymosan as described before.

oxidase system. Dismutation of  $O_2^-$  by SOD completely abrogated the production of NO. Furthermore, the time course of the lucigenin-dependent chemiluminescence as an indicator of  $O_2^-$  synthesis correlates with the amount of nitrite produced in the assay.

#### 3.2. Induction of NO by phagocytic cells during the respiratory burst

Upon challenge with a number of different soluble or particulate agonists, i.e. PMA or zymosan, phagocytes generate a common defence reaction termed the respiratory burst. During this response, a host of reactive oxygenated products is transiently released in large amounts, and consumption of oxygen and glucose is upregulated in these cells [12]. The first step in the reaction cascade of the respiratory burst is the activation of the membrane bound NADPH-oxidase that reduces molecular oxygen to  $O_2^-$ . In order to ascertain that the amounts of  $O_2^-$  produced by phagocytes were sufficient to oxidize the OH-Arg used in the experiments, increasing amounts of OH-Arg were added to murine bone marrow-derived macrophages (BMMΦ) treated with zymosan. The results in Fig. 2 show a linear relationship extending to levels 10 times higher as that employed in all the other experiments (100 µM), suggesting that the amounts of  $O_2^-$  produced during the respiratory burst were not a limiting factor. In addition, as shown in Fig. 3A, we compared the respiratory bursts induced by zymosan in BMMΦ from iNOS-deficient mice and heterozygous littermates. The levels of  $O_2^-$  induced in the iNOS<sup>-/-</sup>BMMΦ were consistently higher than that in littermates. Addition of SOD eliminated the luminescence. Nitrite synthesis in the presence of OH-Arg was measured in a parallel experiment. The zymosan-dependent respiratory burst clearly induces the denitrication of OH-Arg. SOD suppresses the detectable nitrites almost to background levels. The slight increment of nitrites found in the cultures with OH-Arg is most likely due to the constitutive  $O_2^-$  synthesis by resting BMMΦ. Most importantly, the differences in NO synthesis between both BMMΦ populations are insignificant (Fig. 3B).

Next we examined purified human blood monocytes and granulocytes in the same experimental protocol as used above for the BMMΦ. The results are depicted in Fig. 4. In both types of cells, stimulation of the respiratory burst with PMA

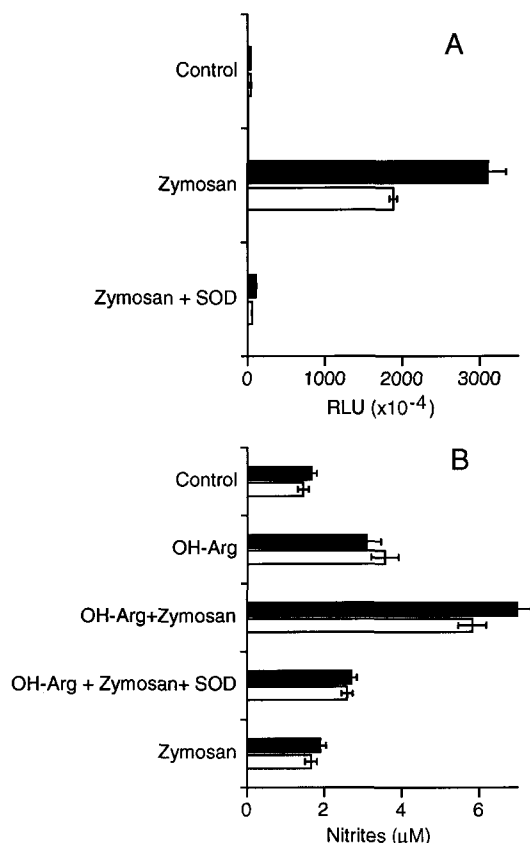


Fig. 3. Induction of the respiratory burst and NO by the  $\text{O}_2^-$  mediated oxidation of OH-Arg in BMMΦ. (A) Zymosan induced chemiluminescence was measured in BMMΦ from normal (open bars) and iNOS knock out mice (closed bars). Values represent the integral cpm of 2 h. (B) BMMΦ ( $10^6/\text{ml}$ ) were cultured for 2 h in the presence of the indicated substances as described before.

(Fig. 4A) or zymosan (Fig. 4B) led to OH-Arg-dependent production of NO, with the same sensitivity to inhibition by SOD. The magnitude of the  $\text{O}_2^-$  response to PMA was greater than to zymosan (data not shown).

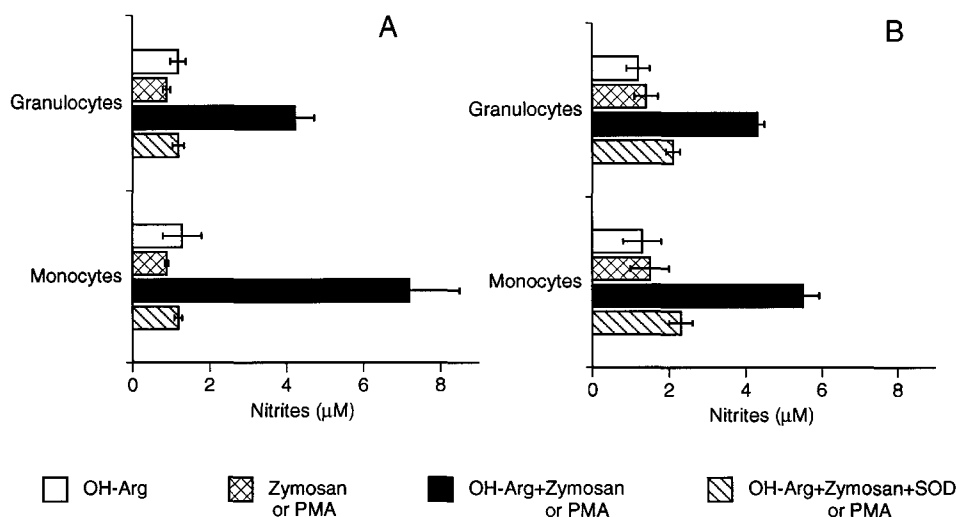


Fig. 4. Synthesis of NO by the  $\text{O}_2^-$  mediated oxidation of OH-Arg in human blood phagocytes. Cells were cultured for 2 h in the presence of (A) PMA or (B) zymosan to trigger the respiratory burst as described in Section 2.

### 3.3. Influence of arginine on the induction of NO by the respiratory burst

$\text{O}_2^-$  is synthesized in the plasma membrane of the phagocytic cells [12]. In order to examine if OH-Arg is oxidized within the cell or in the pericellular environment/endocytic vacuoles, we measured the synthesis of NO upon the zymosan-induced respiratory burst in the presence of different amounts of arginine. The uptake of arginine and OH-Arg depends on the same  $\text{y}^+$  cationic amino acid transport system [13,14]. Extracellular arginine should therefore compete with uptake of OH-Arg into the cell, and decrease intracellular NO synthesis. As shown in Fig. 5, addition of increasing amounts of arginine has no influence on the oxidation of OH-Arg. Thus, oxidation appears to take place independently of uptake into the cell, i.e. presumably in the pericellular environment, the endocytic vacuoles or in the zymosan-induced phagosome.

## 4. Discussion

During the biosynthesis of NO, arginine is first oxidized to OH-Arg. A proportion of this product is further metabolized to NO and citrulline whereas a considerable amount of OH-Arg is secreted together with NO as an adduct [7]. OH-Arg is a strong inhibitor of arginase [15,16] an enzyme constitutively present in macrophages [17]. Several biological effects have been ascribed to OH-Arg including modulation of blood pressure and tumor cell cytostasis [3,14]. Although the oxidation of arginine to OH-Arg is exclusively performed by NOS, the second oxidative step, the conversion of OH-Arg into NO and citrulline, can also be performed by other oxidases/oxidative products [3,18]. Recently, it has been shown that  $\text{O}_2^-$  can mediate cleavage of *N*-hydroxyguanidines resulting in the formation of NO [4]. Moreover, radiolytically induced  $\text{O}_2^-$  can cleave OH-Arg [5]; the products of this reaction are NO and a cyclized carbodiimide, without the concomitant formation of citrulline.

In confirmation of previous studies, we employed the xanthine/xanthine oxidase system for the production of  $\text{O}_2^-$  and to demonstrate its capability to cleave OH-Arg for the synthesis of NO (Fig. 1). The conversion of  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  by

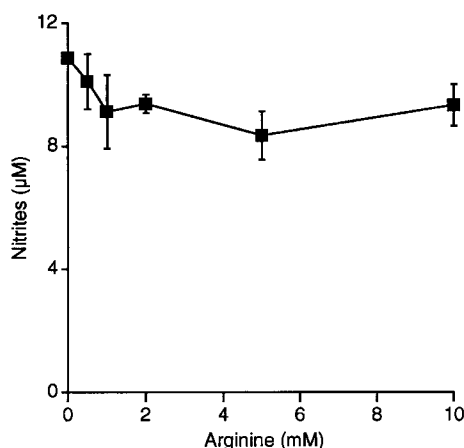


Fig. 5. Synthesis of NO by the respiratory burst in BMMΦ in the presence of different arginine concentrations. BMMΦ were cultured for 2 h in arginine free HEPES-DMEM supplemented with 0.1 mM OH-Arg and the indicated amounts of arginine.

SOD completely arrests the lucigenin-dependent chemiluminescence and the synthesis of NO indicating that  $O_2^-$  is the reactive agent. This result is in partial contrast to two published studies in which SOD increased the NOS mediated synthesis of NO [19,20]. However, in one of these reports, very large amounts of SOD (10 000 U/ml) were used, opening the possibility of contamination with other enzymatic activities. Both studies did not exclude the contamination of SOD with endotoxin that may upregulate the inflammatory cells in their synthetic activity. In our own experiments, endotoxin free SOD showed no influence on the NO synthesis in BMMΦ triggered by *B. burgdorferi* [21] but the impure preparation of SOD upregulated NO synthesis (unpublished data).

The  $O_2^-$  produced during the induction of a respiratory burst in several different types of cells (Figs. 2 and 3) was capable to produce NO by the cleavage of OH-Arg. The reaction is not iNOS dependent as murine BMMΦ of iNOS-deficient mice are able to produce NO at levels similar to cells from wild type mice (Fig. 2). Although we determine relatively low levels of NO in our cellular assays (below 10 µM), these are reached more rapidly than in the reaction mediated by iNOS. The  $O_2^-$  mediated oxidation of OH-Arg possibly induces higher concentrations of the free reactive NO radical. The amount of NO produced is not limited by the generated  $O_2^-$  because, as shown in Fig. 4, concentrations of 0.1–1 mM OH-Arg, i.e. far higher than the physiological levels, kept the reaction linear. At concentrations below 0.1 mM OH-Arg, the production of NO was lower than expected probably because the huge excess of  $O_2^-$  can transform part of the NO in peroxynitrite, a product undetectable by the Griess reagent. Because of the short lifetime of these molecular species, the distribution of nitrogen oxides is difficult to measure. Preliminary attempts to estimate the peroxynitrites as luminol dependent luminescence [22] gave only a transient short signal in the first 30 s of the measurement (data not shown). More likely, the presence of OH-Arg stabilizes NO as an OH-Arg-NO adduct [7] blocking its accessibility to  $O_2^-$  to form peroxynitrites. The linearity observed at higher concentrations of OH-Arg supports this hypothesis.

The addition of low amounts of SOD completely abrogates

the capability of the phagocytes to produce NO. As SOD is unable to diffuse through the plasma membrane of the  $O_2^-$  producing cells, these data suggest that oxidation of OH-Arg takes place in the pericellular space, the endocytic vacuoles or in the phagosomes. This assumption is further supported by our observation that increasing amounts of arginine do not significantly inhibit NO production from OH-Arg (Fig. 5).

In conclusion, we have shown that cells without expression of NOS can produce NO through a respiratory burst if OH-Arg is supplied by neighbouring cells or by the microcirculation. The co-localization of NO and  $O_2^-$  synthesis in the same compartment is consistent with a possible physiological role of this alternative pathway of NO synthesis in the inflammatory and cytotoxic effector functions of the phagocytes.

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## References

- [1] Kröncke, K.-D., Fehsel, K. and Kolb-Bachofen, V. (1995) *Biol. Chem. Hoppe-Seyler* 376, 327–343.
- [2] Marletta, M.A. (1993) *J. Biol. Chem.* 268, 12231–12234.
- [3] Schott, C.A., Bogen, C.M., Vetrovsky, P., Verton, C.C. and Stoclet, J.C. (1994) *FEBS Lett.* 341, 203–207.
- [4] Sennequier, N., Boucher, J.-L., Battioni, P. and Mansuy, D. (1995) *Tetrahedron Lett.* 36, 6059–6062.
- [5] Everett, S.A., Dennis, M.F., Patel, K.B., Stratford, M.R.L. and Wardman, P. (1996) *Biochem. J.* 317, 17–21.
- [6] Hecker, M., Schott, C., Bucher, B., Busse, R. and Stoclet, J.-C. (1995) *Eur. J. Pharmacol.* 275, R1–R3.
- [7] Hecker, M., Boese, M., Schini-Kerth, V.B., Mülsch, A. and Busse, R. (1995) *Proc. Natl. Acad. Sci. USA* 92, 4671–4675.
- [8] MacMicking, J.D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D.S., Trumbaur, M., Stevens, K., Xie, Q., Sokol, K., Hutchinson, N., Chen, H. and Mudgett, J.S. (1995) *Cell* 81, 641–650.
- [9] Munder, P.G., Modolell, M. and Wallach D.F.H. (1971) *FEBS Lett.* 15, 191–196.
- [10] Mollinedo, F., Nieto, J.M. and Andreu, J.M. (1989) *Mol. Pharmacol.* 36, 547–555.
- [11] Minkenberg, I. and Ferber, E. (1984) *J. Immunol. Methods* 71, 61–65.
- [12] Robinson, J.M. and Badway, J.A. (1994) in: *Macrophages-Pathogen Interactions* (Zwilleng, B.S. and Einsenstein, T.K. eds.) pp. 159–195, Dekker, Basel.
- [13] White, M.F. (1995) *Biochim. Biophys. Acta* 822, 355–374.
- [14] Chénais, B., Yapo, A., Lepoivre, M. and Tenu, J.-P. (1993) *Biochem. Biophys. Res. Commun.* 196, 1558–1565.
- [15] Daghigh, F., Fukuto, J.M. and Ash, D.E. (1994) *Biochem. Biophys. Res. Commun.* 202, 174–180.
- [16] Hecker, M., Nematollahi, H., Hey, C., Busse, R. and Racké, K. (1995) *FEBS Lett.* 359, 251–254.
- [17] Hoffmann, F., Kreusch J., Maier, K.-P., Munder, P.G. and Decker, K. (1978) *Biochem. Soc. Trans.* 6, 990–993.
- [18] Puhfahl, R.A., Wishnok, J.S. and Marletta, M.A. (1995) *Biochemistry* 34, 1930–1941.
- [19] Ródenas, J., Mitjavila, M.T. and Carbonell, T. (1995) *Free Radical Biol. Med.* 18, 869–875.
- [20] Hobbs, A.J., Fukuto, J.M. and Ignarro, L.J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 10992–10996.
- [21] Modolell, M., Schaible, U., Rittig, M. and Simon, M.M. (1994) *Immunol. Lett.* 40, 139–146.
- [22] Radi, R., Cosgrove, T.P., Beckmann, J.S. and Freeman, B.A. (1993) *Biochem. J.* 290, 51–57.