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Apocynin inhibits peroxynitrite formation by murine macrophages

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1 Peroxynitrite ($ONOO^-$) the highly reactive coupling product of nitric oxide and superoxide, has been implicated in the pathogenesis of an increasing number of (inflammatory) diseases. At present, however, selective peroxynitrite antagonizing agents with therapeutic potential are not available. Therefore, the NADPH-oxidase inhibitor apocynin (4-hydroxy-3-methoxy-acetophenone) was tested for its ability to inhibit peroxynitrite formation *in vitro*

2 The murine macrophage cell-line J774A.1, stimulated with IFNγ/LPS, was used as a model. Conversion of 123-dihydrorhodamine (123-DHR) to its oxidation product 123-rhodamine was used to measure peroxynitrite production.

3 Stimulated peroxynitrite formation could be completely inhibited by apocynin, by the superoxide scavenger TEMPO as well as by the nitric oxide synthase inhibitor aminoguanidine. Apocynin and aminoguanidine specifically inhibited superoxide and nitric oxide formation respectively as confirmed by measuring lucigenin enhanced chemiluminescence and nitrite accumulation.

4 It is concluded that J774A.1 macrophages produce significant amounts of peroxynitrite, which is associated with nitric oxide production and NADPH-oxidase dependent superoxide formation. The NADPH-oxidase inhibitor apocynin proved to be a potent inhibitor of both superoxide and peroxynitrite formation by macrophages, which may be of future therapeutic significance in a wide range of inflammatory disorders.
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Abbreviations: 123-DHR, 123-dihydrorhodamine; IFNγ, interferon γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysacharide; LUC-CL, lucigenin enhanced chemiluminescence; NO, nitric oxide; ONOO, peroxynitrite; PMA, phorbol-12-myristate-13-acetate; SIN-1, 3-morpholinosydnonimine; TEMPO, 2,2,6,6-tetramethyl-piperidin

Introduction

Peroxynitrite is formed by the diffusion-limited reaction of the free radicals nitric oxide and superoxide (Beckman *et al*., 1990; Blough & Zafiriou, 1985; Squadrito & Pryor, 1995). Inflammatory cells such as neutrophils (McCall *et al*, 1989) and macrophages (Ischiropoulos *et al*., 1992), but also endothelial cells (Kooy & Royall, 1994) can release superoxide and/or NO, potentially leading to peroxynitrite formation. Peroxynitrite is a highly reactive compound with various harmful effects on cells (Muijsers *et al*., 1997) and could therefore be an important microbicidal compound.

In contrast to the possible beneficial effects of peroxynitrite in host defence mechanisms, the anion may have deleterious effects on host tissues. A role for peroxynitrite has been hypothesized in a number of disorders. Examples are human asthma (Saleh *et al*., 1998), acute lung injury (Kooy *et al*., 1995), idiopathic pulmonary fibrosis (Saleh *et al*., 1997), inflammatory bowel disease (Singer *et al*., 1996) and animal models of septic shock (Szabo *et al*., 1994). Inhibition of peroxynitrite formation, by inhibiting either NO or superoxide production, could be a useful aid to limit tissue damage in various circumstances.

Apocynin is a strong inhibitor of the neutrophil oxidative burst (Simons *et al*., 1990). Apocynin inhibits superoxide formation by preventing the assembly of the superoxide-generating enzyme NADPH-oxidase upon activation (Stolk *et al*., 1994). Therefore, apocynin may also be an inhibitor of peroxynitrite formation. This may be of importance because, at present, specific iNOS inhibitors or peroxynitrite scavengers which can be used *in vivo* are not available. Apocynin has previously been demonstrated to be a powerful anti-inflammatory agent in rat models of arthritis ('t Hart *et al*., 1990) and in ulcerative skin lesions in rats ('t Hart *et al*., 1992).

In this study, apocynin was tested on its ability to inhibit peroxynitrite formation by murine macrophages. Therefore, the 123-DHR oxidation assay (Kooy *et al*., 1994) was validated as an index of peroxynitrite formation by immuno-simulated J774A.1 macrophages, providing a tool to screen various compounds on their ability to prevent peroxynitrite formation. Evidence for the dependence of 123-DHR oxidation on both nitric oxide and superoxide was obtained by the use of inhibitors of nitric oxide synthase (aminoguanidine) and by the superoxide scavenger TEMPO (2,2,6,6-tetramethylpiperidin). The specificity of aminoguanidine and apocynin was confirmed by measuring lucigenin-enhanced chemiluminescence and nitrite accumulation respectively.

Part of this study has been previously presented at a meeting of the British Pharmacological Society (Harrogate, December 8–12, 1997; *Br. J. Pharmacol*. (1998) **123**, 41P).

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Methods

Drugs and chemicals

Apocynin (acetovanillone) was obtained from Carl Roth Gmbh (Karlsruhe, Germany) and further purified by recrystallization. Aminoguanidine, LPS (*E. coli* 0111:B4), sulfanilamide, naphthyl-ethylenediamide, lucigenin, PMA and TEMPO (2,2,6,6-tetramethyl-piperidin) from Sigma-Aldrich Chemicals Ltd. Murine recombinant IFNγ from Genetech Ltd. (U.S.A.), 123-DHR from Molecular Probes Europe (Leiden, The Netherlands). $NaHCO_3$ and $NaNO_2$ from Merck (Darmstadt, Germany).

Cell culture

Murine J774A.1 (ATCC, Manassas VA, U.S.A.) macrophages were maintained in RPMI 1640 (supplemented with 10% foetal bovine serum, 10 mM HEPES, 4 mM glutamate, 2 mM pyruvate, 50 μg ml^{-1} gentamycin, penicillin/streptomycin and 100 μM β mercapto-ethanol (all from Gibco-BRL) and cultured at 37°C with 5% CO_2. The cells were passaged every 3 days.

123-DHR oxidation assay

Cells were incubated for 20 h with or without stimuli and inhibitors in the presence of 25 μM 123-dihydrorhodamine (123-DHR) in 96-well microtiter plates in culture medium. After incubation, 123-DHR conversion to 123-rhodamine was measured by fluorimetric analysis at excitation/emission wavelengths of 485 ($\pm$20) and 530 ($\pm$20) nm respectively (Cytofluor 2350, B&L Systems). Fluorescence due to auto-oxidation of 123-DHR was subtracted from the original measurements. Furthermore, oxidation of 123-DHR by SIN-1 (3-morpholinosydnonimine) was measured in the presence of 300 μM apocynin or 1 mM aminoguanidine in culture medium (2 h, 37°C) as described above.

Griess assay

Nitrite concentrations were measured using the Griess reaction (Griess, 1879). One hundred μl of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamide in 5% phosphoric acid) was added to 100 μl of sample medium. After a 10 min incubation at room temperature the optical density was measured at 550 nm using a microplate reader (Biorad). Calibration curves were made with $NaNO_2$ dissolved in the incubation medium.

Chemiluminescence

Macrophages, pre-stimulated overnight with IFNγ (50 u ml^{-1}) and LPS (10 μg ml^{-1}), were incubated in white 96-well (10^6 cells per well 200 μl), flat-bottom microtiter plates, in the presence of lucigenin (400 μM) with and without inhibitors. Macrophages were additionally stimulated with PMA (phorbol-12-myristate-13-acetate, 10 nM). Chemiluminescence was monitored every 3 min for 0.5 s during a 90 min period using a Titertek luminoskan luminometer (TechGen International, Zellik, Belgium). The peak maximum was used to quantify chemiluminescence. After the measurement period, the nitrite concentration in the medium was detected using the Griess assay. Experiments were performed in HBSS (Gibco-BRL) buffered at pH 7.35 with $NaHCO_3$.

Data analysis

All data are expressed as mean$\pm$s.e.mean. The data was statistically analysed using ANOVA followed by *post-hoc* pairwise comparison of the effects of different inhibitor concentrations compared to control levels. Results were considered significantly different at the $P<0.05$ level.

Results

123-DHR oxidation and nitrite accumulation

Upon combined stimulation with recombinant murine IFNγ (50 u ml^{-1}) and LPS (10 μg ml^{-1}) a significant ($P<0.0001$) increase in 123-rhodamine formation and nitrite release compared to unstimulated macrophages was detectable in the medium after 20 h (Figure 1). The nitric oxide synthase inhibitor aminoguanidine dose-dependently inhibited stimulated 123-rhodamine and nitrite accumulation with a logEC_{50} of -3.9 ± 0.06 M (107 μM) and -3.4 ± 0.03 M (438 μM) respectively (Figure 2A,B). Furthermore the superoxide scavenger TEMPO (2,2,6,6-tetramethyl-piperidin) dose dependently inhibited 123-DHR oxidation (logEC_{50} -4.0 ± 0.1 M, 104 μM (Figure 2A). TEMPO did not affect nitric accumulation (Figure 2B). The NADPH-oxidase inhibition apocynin completely inhibited 123-rhodamine accumulation (logEC_{50} $-3.7\pm$ 0.03 M (165 μM) (Figure 2A), whereas nitrite concentrations were practically unaffected (Figure 2B).

123-DHR oxidation by SIN-1

Neither apocynin (300 μM) nor aminoguanidine (1 mM) inhibited 123-DHR oxidation by the peroxynitrite donor SIN-1 (3-morpholinosydnonimine) which releases equimolar amounts of nitric oxide and superoxide (Figure 3).

Lucigenin-enhanced chemiluminescence

Lucigenin-enhanced chemiluminescence (LUC-CL) was measured in cells pre-stimulated with IFNγ and LPS overnight and additionally stimulated with PMA (10 nM) just prior to

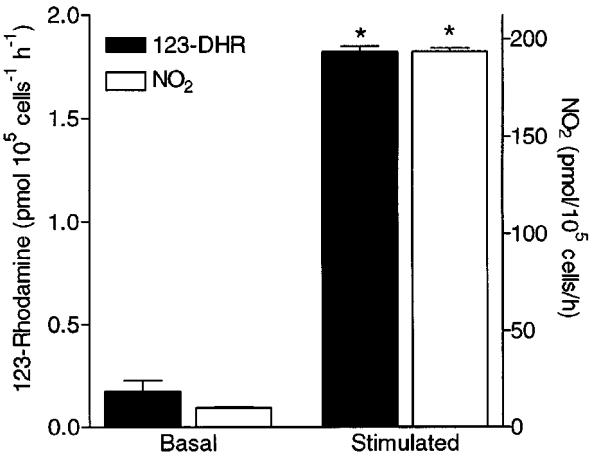

Figure 1 Stimulated 123-DHR oxidation and nitrite accumulation. Effect of IFNγ (50 u ml^{-1}) and LPS (10 μg ml^{-1}) on 123-DHR oxidation and nitrite accumulation by J774A.1 macrophages compared to unstimulated cells, (10^6 cells ml^{-1}, incubated for 20 h). Data represent mean$\pm$s.e.mean of six wells from three independent experiments. *Statistically significant difference ($P<0.0001$) compared with control.

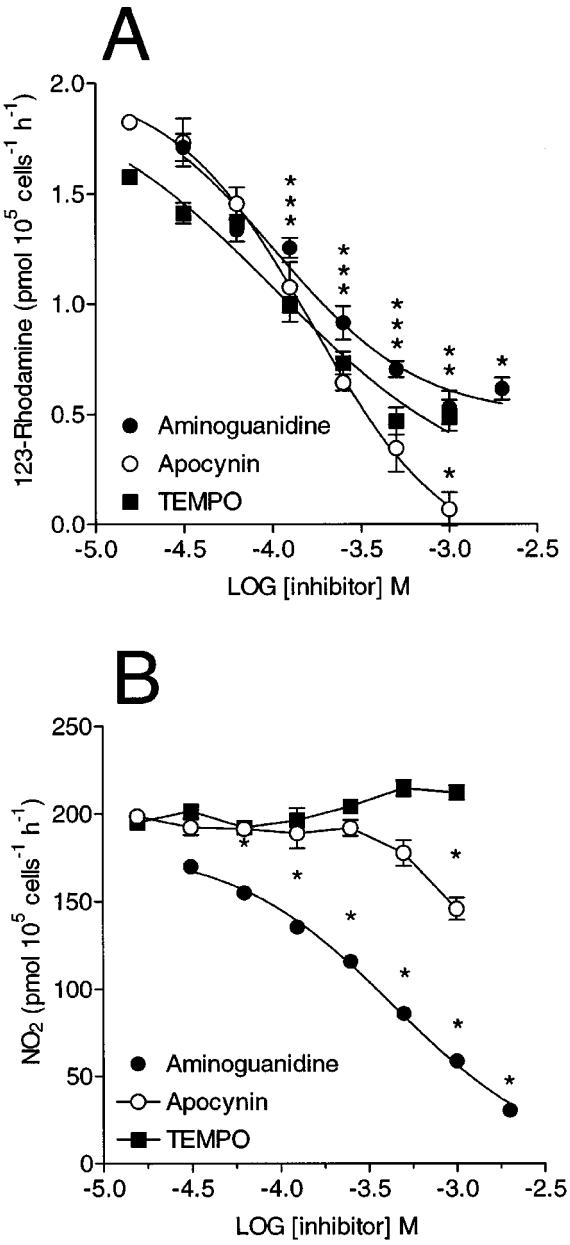

Figure 2 Inhibition of 123-DHR oxidation and nitrite accumulation. Effect of increasing concentrations of aminoguanidine, apocynin and TEMPO on stimulated (IFNγ/LPS 50 u ml^{-1}, 10 μg ml^{-1}) 123-DHR oxidation and nitrite accumulation (B) by J774A.1 macrophages during 20 h of incubation. Data represent mean$\pm$s.e.mean of six wells from three independent experiments. *Statistically significant difference ($P<0.0001$) compared with control.

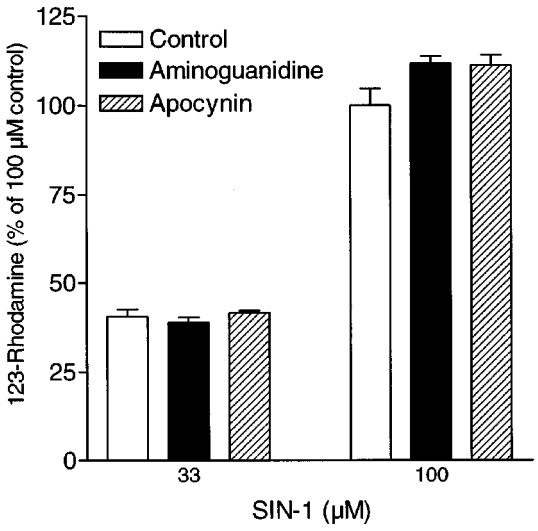

Figure 3 SIN-1 mediated 123-DHR oxidation. Effect of 300 μM apocynin and 1 mM aminoguanidine on 123-DHR oxidation by the peroxynitrite donor SIN-1 (33 and 100 μM) in cell-culture medium compared to control incubations. Data represent mean$\pm$s.e.mean of four replicate wells.

chemiluminescence measurements. Apocynin dose dependently inhibited LUC-CL (Figure 4A) with a logEC_{50} -3.5 ± 0.1 M (309 μM). Aminoguanidine, however, did not affect LUC-CL (Figure 4A). Nitrite measurements, done after the 90 min chemiluminescence incubation (Figure 4B), revealed that aminoguanidine inhibited NO output with a log EC_{50} of 4.7 ± 0.1 (16 μM) during this short incubation period. Apocynin did not affect nitrite accumulation in this system, not even at the highest concentration used (2 mM).

Discussion and conclusions

It is important to stress that 123-DHR oxidation can only be attributed to peroxynitrite formation when shown to be dependent on both nitric oxide and superoxide. 123-DHR oxidation by stimulation human neutrophils, for example, is largely dependent on hydrogen peroxide formation and peroxidase activity (Henderson & Chappell, 1993) showing that 123-DHR is certainly not a specific probe for peroxynitrite.

Inhibition of nitric oxide synthase by aminoguanidine revealed that the release of reactive oxygen species alone cannot explain 123-DHR oxidation measured in stimulated macrophages. Similarly, scavenging (TEMPO) or inhibition of the formation of superoxide (apocynin) showed that 123-DHR is insensitive to nitric oxide. Hence, 123-DHR oxidation by immunostimulated J774A.1 macrophages is mainly dependent on the simultaneous release of nitric oxide and superoxide and is therefore attributed to peroxynitrite considering the likelihood of the interaction between the two precursors (Huie & Padmaja, 1993). Moreover, the present data suggest that NADPH-oxidase is the main source of superoxide leading to peroxynitrite formation in J774A.1 macrophages since 123-DHR oxidation was completely inhibited by the NADPH-oxidase inhibitor apocynin.

Neither apocynin nor aminoguanidine are scavengers of peroxynitrite, superoxide or nitric oxide since neither of the compounds inhibited SIN-1 mediated 123-DHR oxidation. SIN-1 is a donor or equimolar amounts of superoxide and nitric oxide and consequently of peroxynitrite (Darley-Usmar *et al*., 1992). Specificity of the inhibitors was further supported by lucigenin-enhanced chemiluminescence, which detects reactive oxygen species, but is insensitive to peroxynitrite and hypochlorous acid (Aniansson *et al*., 1984). In this study, lucigenin-enhanced chemiluminescence could be completely blocked by apocynin, but was unaffected by aminoguanidine. The other way around, nitrite accumulation during these incubations was completely inhibited by aminoguanidine and was unaffected by apocynin.

Theoretically, inhibition of superoxide formation would enhance nitrite accumulation since NO is then no longer scavenged by superoxide leading to peroxynitrite formation. Nitrite measurements in the supernatant of stimulated macrophages however showed that inhibition of superoxide does not increase nitrite accumulation. There are two possible

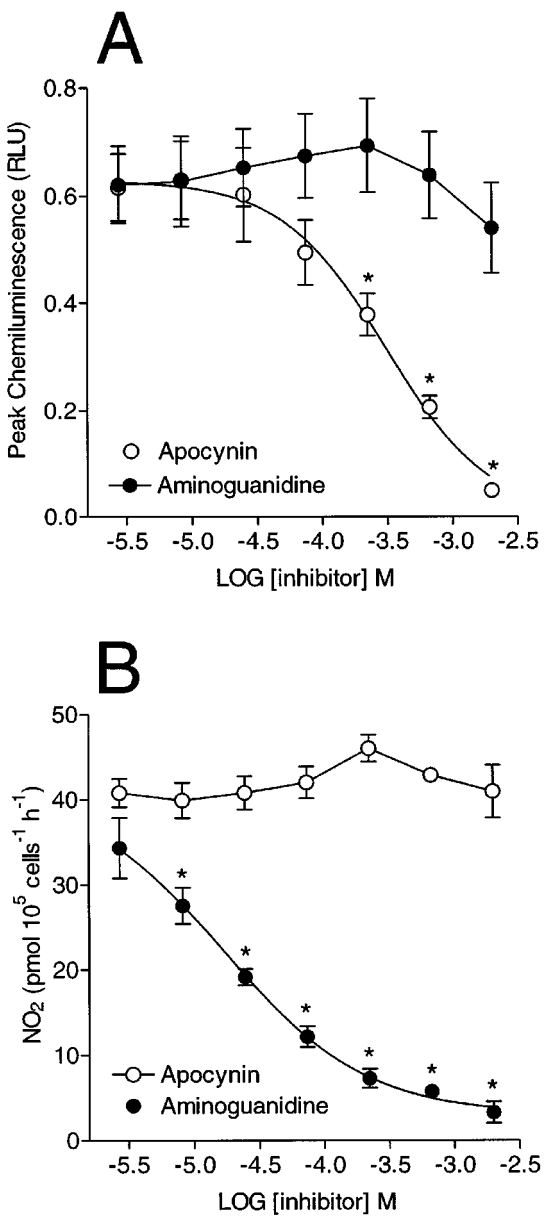

Figure 4 Lucigenin-enhanced chemiluminescence. Effect of apocynin and aminoguanidine on peak lucigenin-enhanced chemiluminescence (A) and nitrite accumulation after 90 min (B) by J774A.1 macrophages pre-stimulated with IFNγ (50 u ml^{-1}) and LPS (10 μg ml^{-1}) for 20 h and additionally stimulated with PMA just prior to chemiluminescence measurements (10 nM). Data represent mean$\pm$s.e.mean of six replicate wells from two independent experiments. *Statistically significant difference ($P<0.0001$) compared with control.

explanations for this discrepancy. Either peroxynitrite mainly decays into nitrite in the present system, leaving the nitrite accumulation unaltered, or the amount of NO consumed by the reaction with superoxide is too low to be detected by differences in nitrite accumulation. Likewise, inhibition of NO formation did not increase lucigenin-enhanced chemiluminescence. It is not unlikely, however, that the additional PMA stimulus led to a relative overproduction of superoxide during the chemiluminescence measurements, which would make the difference in the chemiluminescence signal too small to detect.

The exact metabolic fate of peroxynitrite once formed in a biological environment is not exactly clear at present. For example, several studies have shown an important role for carbon dioxide in peroxynitrite mediated reactions (Bonini *et al*., 1999; Radi *et al*., 1999; Uppu *et al*., 1996). Furthermore, the activity/concentration of cellular antioxidant mechanisms are likely to play a pivotal role in downstream events initiated by peroxynitrite. Therefore, the reaction conditions for peroxynitrite and 123-DHR in the presence of or inside cells may differ significantly from those in cell free systems. For this reason, calibration of the 123-DHR assay to exact concentrations of peroxynitrite as detected in cell free systems is questionable. Moreover, it is unknown whether peroxynitrite itself or downstream metabolites of the anion are responsible for the 123-DHR oxidation detected in immunostimulated macrophages.

Although the evidence for peroxynitrite formation *in vivo* is convincing, recent studies have suggested that other reactive nitrogen species may be as, or even more important mediators of oxidative tissue modifications during inflammatory processes (Van der Vliet *et al*, 1999). The formation of the putative peroxynitrite footprint 3-nitrotyrosine, for example, has been demonstrated to be readily catalyzed by myeloperoxidase with hydrogen peroxide and nitrite as substrates (Eiserich *et al*., 1998). Therefore, nitric oxide formation as such may not be an essential factor in reactive nitrogen species mediated tissue alterations during inflammation. In contrast, superoxide and its downstream metabolics appear to have a pivotal role in all pathways leading to reactive nitrogen species described above. Consequently, limiting superoxide production by apocynin may prevent the formation of peroxynitrite as well as other reactive nitrogen species.

The present data clearly show that murine J774A.1 macrophages stimulated with IFNγ and LPS release high amounts of NO and superoxide, most probably leading to peroxynitrite formation. Moreover, stimulated 123-DHR oxidation by J774A.1 macrophages is dependent on both superoxide and nitric oxide and is therefore likely to be dependent on peroxynitrite or downstream metabolites of the compound. This is the first time that the NADPH-oxidase inhibitor apocynin is shown to be a potent inhibitor of 123-DHR oxidation by murine macrophages *in vitro*. Apocynin has potential value as a tool to limit peroxynitrite formation in inflammatory conditions *in vivo*. Considering the lack of specific iNOS inhibitors that can be used therapeutically, apocynin could have an additive value because it is not only an inhibitor of superoxide release, but is also a powerful inhibitor of peroxynitrite formation.

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References

ANIANSSON, H., STENDAHL, O. & DAHLGREN, C. (1984). Comparison between luminol- and lucigenindependent chemiluminescence of polymorphonuclear leukocytes. *Acta. Pathol. Microbiol. Immunol. Scand.,* **92,** 357–361.

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. of Sci. U.S.A.,* **87,** 1620–1624.

BLOUGH, N.V. & ZAFIRIOU, O.C. (1985). Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline aqueous solution. *Inorg. Chem.,* **24,** 3502 – 3504.

BONINI, M.G., RADI, R., FERRER-SUETA, G., FERREIRA, A.M. & AUGUSTO, O. (1999). Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J. Biol. Chem.,* **274,** 10802 – 10806.

DARLEY-USMAR, V.M., HOGG, N., O'LEARY, V.J., WILSON, M.T. & MONCADA, S. (1992). The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Rad. Res. Com.,* **17,** 9 – 20.

EISERICH, J.P., HRISTOVA, M., CROSS, C.E., JONES, A.D., FREEMAN, B.A., HALLIWELL, B. & VAN DER VLIET, A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature,* **391,** 393 – 397.

GRIESS, P. (1879). *Chem. Ber.,* **12,** 426.

HART, 'T, B.A., ELFERINK, J.G. & NIBBERING, P.H. (1992). Effect of apocynin on the induction of ulcerative lesions in rat skin injected with tubercle bacteria. *Int. J. Immunopharmacol.,* **14,** 953 – 961.

HART, 'T, B.A., SIMONS, J.M., KNAAN-SHANZER, S., BAKKER, N.P. & LABADIE, R.P. (1990). Antiarthritic activity of the newly developed neutrophil oxidative burst antagonist apocynin. *Free. Rad. Biol. Med.,* **9,** 127 – 131.

HENDERSON, L.M. & CHAPPEL, J.B. (1993). Dihydrorhodamine 123: a fluorescent probe for superoxide generation? *Eur. J. Biochem.,* **217,** 973 – 980.

HUIE, R.E. & PADMAJA, S. (1993). The reaction of no with superoxide. *Free Rad. Res. Com.,* **18,** 195 – 199.

ISCHIROPOULOS, H., ZHU, L. & BECKMAN, J.S. (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.,* **298,** 446 – 451.

KOOY, N.W. & ROYALL, J.A. (1994). Agonist-induced peroxynitrite production from endothelial cells. *Arch. Biochem. Biophys.,* **310,** 352 – 359.

KOOY, N.W., ROYALL, J.A., ISCHIROPOULOS, H. & BECKMAN, J.S. (1994). Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Rad. Biol. Med.,* **16,** 149 – 156.

KOOY, N.W., ROYALL, J.A., YE, Y.Z., KELLY, D.R. & BECKMAN, J.S. (1995). Evidence for in vivo peroxynitrite production in human acute lung injury. *Am. J. Respir. Crit. Care Med.,* **151,** 1250 – 1254.

MCCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. *Biochem. J.,* **261,** 293 – 296.

MUIJSERS, R.B.R., FOLKERTS, G., HENRICKS, P.A.J., SADEGHI-HASHJIN, G. & NIJKAMP, F.P. (1997). Peroxynitrite: A two-faced metabolite of nitric oxide. *Life Sci.,* **60,** 1833 – 1845.

RADI, R., DENICOLA, A. & FREEMAN, B.A. (1999). Peroxynitrite reactions with carbon dioxide-bicarbonate. *Methods Enzymol.,* **301,** 353 – 367.

SALEH, D., BARNES, P.J. & GIAID, A. (1997). Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care. Med.,* **155,** 1763 – 1769.

SALEH, D., ERNST, P., LIM, S., BARNES, P.J. & GIAID, A. (1998). Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J.,* **12,** 929 – 937.

SIMONS, J.M., 'T HART, B.A., VAI CHING, T.R.A.M., VAN DIJK, H. & LABADIE, R.P. (1990). Metabolic activation of natural phenols into selective oxidative burst antagonists by activated human neutrophils. *Free Rad. Biol. Med.,* **8,** 251 – 258.

SINGER, I.I., KAWKA, D.W., SCOTT, S., WEIDNER, J.R., MUMFORD, R.A., RIEHL, T.E. & STENSON, W.F. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology,* **111,** 871 – 885.

SQUADRITO, G.L. & PRYOR, W.A. (1995). The formation of peroxynitrite in vivo from nitric oxide and superoxide. *Chem. Biol. Interact.,* **96,** 203 – 206.

STOLK, J., HILTERMANN, T.J., DIJKMAN, J.H. & VERHOEVEN, A.J. (1994). Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am. J. Respir. Cell. Mol. Biol.,* **11,** 95 – 102.

SZABO, C., SOUTHAN, G.J. & THIEMERMANN, C. (1994). Beneficial effects and improved survival in rodent models of septic shock and S-methyl-isothiourea sulfate, a novel, potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 12472.

UPPU, R.M., SQUADRITO, G.L. & PRYOR, W.A. (1996). Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.,* **327,** 335 – 343.

VAN DER VLIET, A., EISERICH, J.P., SHIGENAGA, M.K. & CROSS, C.E. (1999). Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? *Am. J. Respir. Crit. Care. Med.,* **160,** 1 – 9.

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