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Carbon monoxide modulates the response of human basophils to $Fc_\varepsilon RI$ stimulation through the heme oxygenase pathway

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Abstract

We report the effects of exogenous and endogenous carbon monoxide (CO) on the immunological activation of human basophils. Hemin (1 – 100 μM), a heme oxygenase substrate analogue, significantly increased the formation of bilirubin from partially purified human basophils, thus indicating that these cells express heme oxygenase. This effect was reversed by preincubating the cells for 30 min with Zn-protoporphyrin IX (100 μM), a heme oxygenase inhibitor. Hemin (100 μM) also decreased immunoglobulin G anti-Fc_ε (anti-IgE)-induced activation of basophils, measured by the expression of a membrane granule-associated protein, identified as cluster differentiation protein 63 (CD63), and by histamine release. These effects were reversed by Zn-protoporphyrin IX (100 μM), by oxyhemoglobin (HbO_2), a CO scavenger (100 μM), and by 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ), an inhibitor of the soluble guanylyl cyclase (100 μM). Exposure of basophils to exogenous CO (10 μM for 30 min) also decreased their activation, while nitrogen (N_2) was ineffective. HbO_2 and ODQ reversed the inhibition, reversing both membrane protein CD63 expression and histamine release to basal values. Both hemin and exogenous CO significantly raised cGMP levels in basophils and blunted the rise of calcium levels caused by immunological activation. This study suggests that CO increases cGMP formation, which in turn induces a fall in intracellular Ca^{2+} concentration, thereby resulting in the inhibition of human basophil activation.

Keywords: Heme oxygenase; Basophil, human; Histamine; CD63; cGMP

1. Introduction

An increase or a decrease in the expression of heme oxygenase may represent a new pathophysiological mechanism capable of modulating basophil-mediated, IgE-dependent immune responses through the endogenous generation of CO. Among the inflammatory cells, circulating basophils are of paramount relevance in the primary and late-phase reaction of immunoglobulin E (IgE)-mediated immune response, in that the cross-linking of membrane-bound IgE molecules by the specific antigen causes the release of pre-stored and newly generated pro-inflammatory mediators through a calcium and cyclic nucleotide-mediated process (Foreman, 1984). Several endogenous autacoids can modulate the immune response of human basophils; among them, histamine has been shown to inhibit the anaphylactic secretion of pre-stored mediators from these cells, in a way which is antagonized by histamine H_2 receptor antagonists (Lichtenstein and Gillespie, 1975). The presence of functionally active histamine H_2 receptors on the basophil surface accounts for this inhibitory effect (Lichtenstein and Gillespie, 1975). More recently, two gaseous autacoids, nitric oxide (NO) and carbon monoxide (CO), have been found to act as intracellular signalling molecules in a variety of biological systems (Maines, 1996; Tomlinson and Willoughby, 1999). In some inflammatory cells, NO and CO share a similar action. In isolated rat and guinea pig serosal mast cells, the NO donor sodium nitroprusside inhibits the immunological and non-immunological release of histamine (Salvemini et al., 1991). The inhibitory effect of NO was confirmed by Iikura et al. (1998) in crude rat peritoneal mast cells and was potentiated by increasing the bioavailability of NO by *N*-acetylcysteine. The addition of interferon-γ to mouse peritoneal cells led to NO synthesis and was associated with decreased IgE-mediated mast cell degranulation.

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The NO donor *S*-nitrosoglutathione inhibited the degranulation of purified rat peritoneal mast cells stimulated by either IgE cross-linking or calcium ionophore (Koranteng et al., 2000). In human basophils, immunoglobulin G anti-human Fc_ε (anti-IgE)-induced histamine release was inhibited by combining sodium nitroprusside and *N*-acetylcysteine (Iikura et al., 1998). Carbon monoxide, which acts on guanylyl cyclase, the same intracellular target as NO, has been also shown to down-regulate the release of histamine induced by compound 48/80 from rat serosal mast cells (Di Bello et al., 1998) and from mast cells isolated from actively sensitised guinea pigs (Ndisang et al., 1999). However, no information is available on the effect of CO on the immunological activation of human basophils. The analogy between NO and CO prompted us to study the effect of endogenous and exogenous CO on the anti-IgE induced activation of human basophils.

2. Materials and method

2.1. Preparation of basophil-rich leukocyte samples

Twenty healthy donors were recruited in the Transfusion Unit of Careggi General Hospital (Florence). The subjects did not suffer from allergic diseases and had not taken any drug during the previous 4 weeks. They gave informed consent to their enrollment in this study. About 400 ml of venous blood was collected from each person; 64 ml of a citrate solution was added as anticoagulant. The blood was centrifuged at 3500 rpm (11 min, 20 °C) in a slow-stop centrifuge (Sorvall RC 12 BP, Kendro Laboratory Products, USA); plasma was removed by an automatic press (NPBI Compomat 64, Fresenius HemoCare, Germany). After 24 h of gentle agitation in a platelet incubator (Helmer PSF84, Noblesville, USA) at 22 °C to reduce cell stress, the buffy coat was centrifuged at 900 rpm (9 min, 20 °C); platelet-rich plasma was removed by the same automatic press. Thirty milliliters of the residual leukocyte-rich preparation was diluted 1:4 with a buffer of the following composition: HEPES 20 mM, NaCl 130 mM, KCl 5 mM, sodium heparin 5 IU/ml, bovine serum albumin 1.5 mg/ml, at pH 7.4 (washing buffer), according to the method of Miroli et al. (1986), with some modification (Bani et al., 2002). Aliquots of 10 ml were then carefully layered over 10 ml of Ficoll-Paque in 30-ml conical tubes (25-mm diameter) and centrifuged at room temperature at $420 \times g$. After removal of supernatant plasma, the basophil-rich Ficoll-Paque layer was separated, discarding the neutrophil-rich buffy coat. The suspension was washed twice with the washing buffer and centrifuged at $200 \times g$ at 20 °C for 10 min. The pellets were then resuspended in a calcium-free, maintenance buffer composed of HEPES 20 mM, NaCl 130 mM, KCl 5 mM, Na_3EDTA 5 mM, bovine serum albumin 1.5 mg/ml, pH 7.4, and further processed as described below. Upon isolation, cell viability, determined by Trypan blue exclusion, was always greater than 95%. The procedure used, due to the low handling of basophils, also prevented their aspecific activation, as might have occurred with high-purification procedures, such as specific antibody-coated magnetic beads. Before the experiments were started, samples from each basophil-rich leukocyte preparation were challenged for their ability to respond to anti-IgE in the flow cytometric assay described below. Poorly responsive preparations were discarded.

2.2. Treatments

Activation of human basophils was evaluated in buffer containing HEPES 20 mM, NaCl 130 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 0.2 mM, sodium heparin 5 IU/ml, bovine serum albumin 1.5 mg/ml, pH 7.4. According to the method described by VanUffelen et al. (1996), saturated CO stock solutions were freshly prepared before every experiment. CO was bubbled for 30 min through the buffer in a glass bottle, and the CO concentration was measured using the spectrophotometric method described by Chalmers (1991). Briefly, CO was trapped with hemoglobin (Hb) to form carboxyhemoglobin (COHb) and subsequently estimated by dithionite reduction with a spectrophotometric method. The CO content of the buffer after saturation was 1.1 mM. Saturated CO stock solution was then diluted with air-exposed buffer to obtain a CO concentration of 10 μM. The same process was performed by bubbling N_2 through the buffer to obtain a N_2-saturated solution to be used as a control. The leukocyte preparations were divided into three samples, each containing about 10^5 cells: one of them was incubated in a sealed tube with buffer containing 10 μM CO for 30 min, another was incubated with N_2-saturated buffer, and the third one was incubated with buffer in air. Incubation was carried out in a shaking water bath at 37 °C. Some samples were pretreated for 30 min with 100 μM 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) or with 100 μM HbO_2 before exposure to CO. At the end of the incubation, basophils were activated by adding 1 μg/ml anti-IgE antibodies (Sigma, St. Louis, MO, USA) to the sample for 10 min. Use of a basophil-rich leukocyte preparation instead of purified basophils did not affect the specificity of the response. In fact, in our cell suspensions, only basophils possess high-affinity Fc_ε receptors (Thompson et al., 1990) and hence can respond to activation by anti-IgE antibodies. In another set of experiments, basophils were incubated for 1 h with hemin (100 μM), or with hemin (100 μM) in the presence of ODQ (100 μM), or HbO_2 (100 μM). In some experiments, the cell were preincubated for 30 min with Zn-protoporphyrin IX (100 μM) before the addition of hemin (100 μM). At the end of the treatments, the cells were challenged with anti-IgE (1 μg/ml).

2.3. Evaluation of heme oxygenase activity

Basophil-rich leukocyte suspensions were incubated for 1 h with the given concentration of hemin in the presence or

in the absence of Zn-protoporphyrin IX 100 μM. At the end of the incubation, the cells were washed, homogenized and incubated for 30 min at 37 °C with 50 μl rat liver biliverdin reductase (0.42 μg protein in a volume of 200 μl) to convert biliverdin to bilirubin (Llesuy and Tomaro, 1994). The level of bilirubin was measured spectrophotometrically, using a Sigma Diagnostics procedure (Sigma). The method is based on the reaction of total bilirubin with diazotized sulfanilic acid in the presence of dimethylsulfoxide to give azobilirubin, which is measured spectrophotometrically at 560 nm.

2.4. Flow cytometric analysis

Basophil-rich leukocyte pellets were labeled with a saturating concentration of anti-IgE fluorescein isothiocyanate-conjugated antibodies and anti-membrane granule-associated protein, identified as cluster of differentiation 63 (CD63) phycoerythrin-conjugated antibodies. The fluorescent antibodies were incubated with the pellets for 20 min at 4 °C. The cells were then washed with buffer, centrifuged at 200 × *g* for 10 min at room temperature and then resuspended in buffer. After the lysis of residual erythrocytes, the leukocyte suspensions were analyzed with a flow cytometer (Coulter XL, Coulter Cytometry, Hialeah, FL, USA). Since the separation technique provides a leukocyte preparation with no more than 70% basophils, it was necessary to sort the basophil-related events using appropriate electronic gates. Basophils were recognized by their high expression of membrane-bound IgE, which results in a high signal related to fluorescein isothiocyanate fluorescence (emission peak at 530 nm) (Fig. 1, Panel A). IgE-negative cells were then gated out by electronic subtraction (Fig. 1, Panel B). The fluorescent signal of phycoerythrin (emission peak at 575 nm) was used to characterize activated and non-activated cells. Human basophils before activation showed a low expression of CD63 (Fig. 1, Panel B), which was strongly expressed after the activation of the cells (Fig. 1, Panels C and D) (Knol et al., 1991; Sainte-Laudy et al., 1998).

2.5. Histamine release assay

Histamine was measured fluorimetrically using the method of Shore et al. (1959) as modified by Kremzer and Wilson (1961). In the supernatants, *o*-phthaldialdehyde was added directly to the samples after alkaliniza-

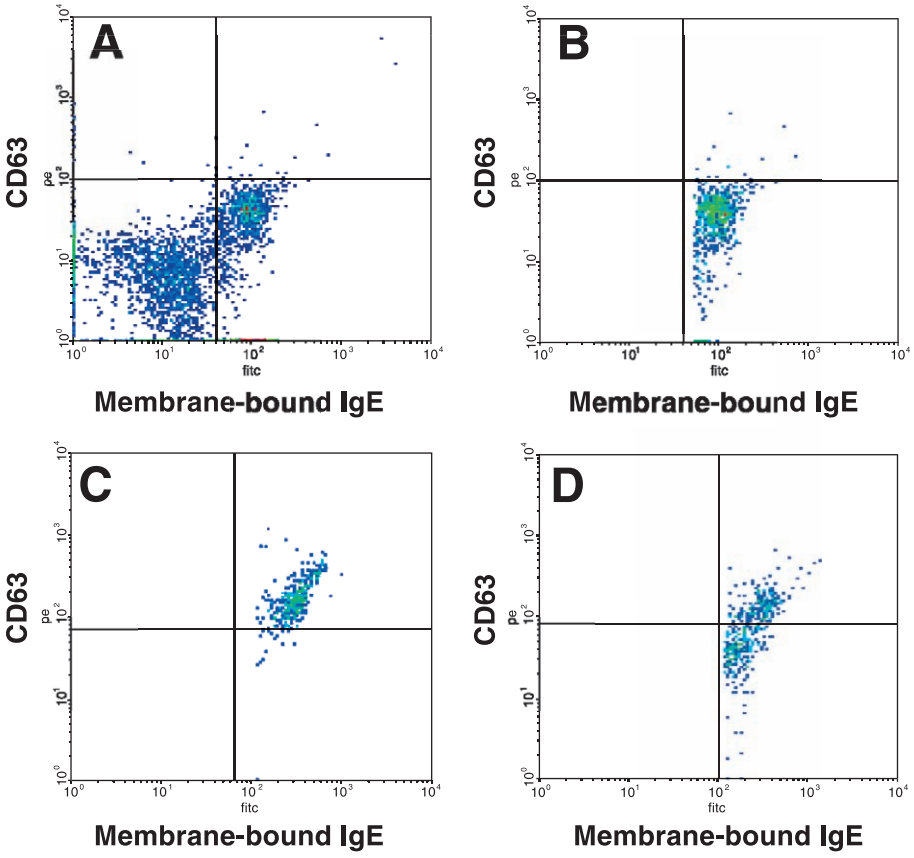

Fig. 1. Flow cytometric evaluation of human basophils. Human basophils at rest show a high expression of membrane-bound IgE and a low expression of CD63 (Panel A). After electronic subtraction of IgE-negative cells (Panels B, C and D), the percentage of activated basophils was calculated under basal conditions (Panel B) and after stimulation (anti-IgE antibodies, 1 μg/ml) both in untreated (Panel C) and treated cells (Panel D, carbon monoxide 10 μM for 30 min).

tion. The same procedure was used for the cells after extraction with 0.1 M HCl, using the method of Bergendorff and Uvnas (1972). Histamine release (supernatant histamine) is expressed as a percentage of the total present in the cell plus supernatants. Spontaneous histamine release ranged between 1% and 5% and was subtracted from all values.

2.6. Evaluation of $[Ca^{2+}]_i$ levels

A basophil-rich suspension was diluted in a buffer of the following composition: HEPES 10 mM, NaCl 140 mM, KCl 3 mM, $MgC1_2$ 0.1 mM, 0.1% glucose, bovine serum albumin 1.5 mg/ml, pH 7.4 and loaded with 3 μM Fura-2 acetoxymethyl ester (Fura 2-AM) for 1 h in a shaking water bath at 37°C. The cell suspension was then centrifuged at 200 × *g*, the supernatant was discarded, and the pellet was washed twice with the same buffer. The cell suspension was divided into three samples, which were incubated with buffer exposed to air, buffer containing 10 μM CO, or buffer exposed to 100% N_2, and the incubation was carried out for 30 min at 37°C. Several aliquots of the cell suspension were placed in quartz cuvettes at 37°C, under constant stirring and stimulated or not with anti-human IgE (1 μg/ml). Cytosolic-free Ca^{2+} levels were determined spectrofluorimetrically according to Tsien et al. (1982), using a spectrofluorimeter (RF5000, Shimadzu, Osaka, Japan) which allows the measurement of both peak values and plateau values. F_{min} was obtained by measuring fluorescence in the presence of 8 mM EGTA (pH 8.5) and F_{max} was obtained by measuring fluorescence in digitonin-lysed basophil samples in the presence of 3 mM Ca^{2+}. A K_d value of 224 nM was used for the apparent dissociation constant of Fura-2 AM (Vasdev et al., 1988).

2.7. Evaluation of cyclic GMP

Different pools of basophil-rich leukocyte samples were incubated for 30 min at 37°C in the presence of 10 μM CO or air and then challenged with 1 μg/ml anti-IgE antibodies. To inhibit phosphodiesterase activity, 3^1-isobutyl-1-methyl xanthine (IBMX, 10 μM) was added to the cells. The concentration of cGMP was determined by means of a radioimmunoassay kit using [^{125}I]-labeled cGMP (Amersham, Bucks, UK). After incubation, 500 μl of 10% trichloroacetic acid was added to the basophil suspensions. The samples were then centrifuged and trichloroacetic acid was extracted with 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichlorotrifluoroethane. The samples were then acetylated with acetic anhydride (Harper and Brooker, 1975) and the amount of cGMP was measured in the aqueous phase. The values are expressed as fmol of cGMP per mg of proteins. The protein concentration was determined according to Bradford (1976).

2.8. Electron microscopy

Basophil-rich leukocyte suspensions were pelleted by centrifugation, fixed in cold 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 60 min at room temperature and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at room temperature for 1 h. The pellets were then dehydrated in graded acetone, passed through propylene oxide and embedded in Epon 812. Semithin sections, 2 μm thick, were stained with toluidine blue-sodium tetraborate, passed through an ascending series of ethanol, mounted in Permount and viewed under a light microscope to identify basophils, based on the metachromatic staining of their granules. Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV.

2.9. Drugs used

Pure 99.9% CO and 99.9% N_2 were obtained as gas from SOL (Italy). HEPES, EDTA, anti-human IgE antibodies and anti-human IgE fluorescein isothiocyanate-conjugate antibodies were obtained from Sigma (Milano, Italy); anti-human CD63 phycoerythrin-conjugate antibodies were obtained from Coulter (Milano, Italy). NaCl, KCl, NH_4Cl, $KHCO_3$ were purchased from Merck (Darmstadt, Germany); bovine serum albumin was bought from Boehring (Germany); heparin was from Parke-Davis (Milano, Italy); [^{125}I]cGMP radioimmunoassay was from Amersham; rat liver biliverdin reductase was from Stress Gen Biotech. (Canada). Oxyhemoglobin was prepared by reduction of bovine hemoglobin with two crystals of sodium hydrosulfite followed by gel filtration with a prepacked disposable column (PD-10, Pharmacia, Uppsala, Sweden), previously equilibrated with 50 mM Tris/HCl at pH 7.4 (Salvemini et al., 1989). The concentration of HbO_2 was determined with a spectrophotometric method using a Perkin Elmer Lambda 5 spectrophotometer at 576 nm wavelength, according to Kondo et al. (1989). 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) was purchased from Tocris Cookson (Langford, Bristol, UK); Zn-protoporphyrin IX (8,13-divinyl-3,7,12,17-tetramethyl-21*H*,23*H*-porphine-2,18-dipropionic acid, zinc derivative) was purchased from Aldrich Chem. (Milwaukee, USA); 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-acetoxymethyl ester (Fura 2-AM) was from Calbiochem (Darmstadt, Germany).

2.10. Statistical analysis

Statistical analysis was performed using SPSS statistic software (release 11.0; SPSS, Chicago, IL, USA). Comparisons of groups were done using Student's *t*-test for unpaired values or Kruskal–Wallis *h*-test, when appropriate. *P* values

equal to or less than 0.05 were considered statistically significant.

3. Results

The leukocyte population used for our experiments was composed of approximately 70% basophils and 30% lymphocytes, with very few leukocytes of the other types, as assessed by flow cytometry (Fig. 1) and electron microscopy (Fig. 2).

Unstimulated preparations of enriched human basophils generated a small but detectable amount of bilirubin in the presence of biliverdin reductase, providing evidence of the presence of the heme oxygenase pathway. Incubation with hemin (1–100 μM), a heme oxygenase 1 inducer, dose dependently increased the generation of bilirubin. This increase was significantly reduced in the presence of Zn-protoporphyrin IX, an inhibitor of heme oxygenase 1, in a dose-dependent way with a maximum effect at 100 μM (Table 1).

Flow cytometric analysis allows quantitative detection of the immunological activation of basophils (Sainte-Laudy et al., 1998), based on double-labeling for membrane-bound IgE, expressed by the entire basophil population, and for membrane protein CD63, expressed by activated basophils only (Knol et al., 1991). Basophils pretreated with hemin showed a reduced expression of CD63 in response to immunological stimulation; the inhibition was reversed by Zn-protoporphyrin IX. A significant release of endogenous histamine occurred from basophils challenged with anti-IgE. Consistent with the inhibition of the expression of the membrane protein CD63, the release of histamine was significantly lower in basophils pretreated with hemin. Blockade of heme oxygenase activity by Zn-protoporphyrin IX, scavenging of CO with HbO_2 and blockade of guanylyl cyclase with ODQ restored both the release of histamine and the expression of membrane protein CD63 to the values of unstimulated basophils (Fig. 3).

Exposure to 10 μM CO for 30 min before antigen challenge gave similar results as pretreatment with hemin.

Table 1
Production of bilirubin by hemin-induced heme oxygenase activity in human basophils

Treatment	Heme oxygenase activity (pg bilirubin/μg protein/h)
Control	340.82 ± 20.12
Hemin 1 μM	527.36 ± 19.30[a]
Hemin 10 μM	737.51 ± 26.39[a]
Hemin 100 μM	1290.76 ± 41.82[b]
Hemin 100 μM + Zn-protoporphyrin IX 10 μM	1027.52 ± 37.45
Hemin 100 μM + Zn-protoporphyrin IX 50 μM	876.92 ± 31.15[c]
Hemin 100 μM + Zn-protoporphyrin IX 100 μM	487.39 ± 27.51[d]

Human basophils were incubated for 1 h with hemin (1–100 μM) or hemin (100 μM) + Zn-protoporphyrin (100 μM, 1 h before hemin). The determination of heme oxygenase activity was carried out as described in Materials and method. The results are presented as means ± S.E.M. of six experiments performed in duplicate.

[a] $P < 0.05$ vs. control.
[b] $P < 0.001$ vs. control.
[c] $P < 0.05$ vs. hemin alone.
[d] $P < 0.001$ vs. hemin alone.

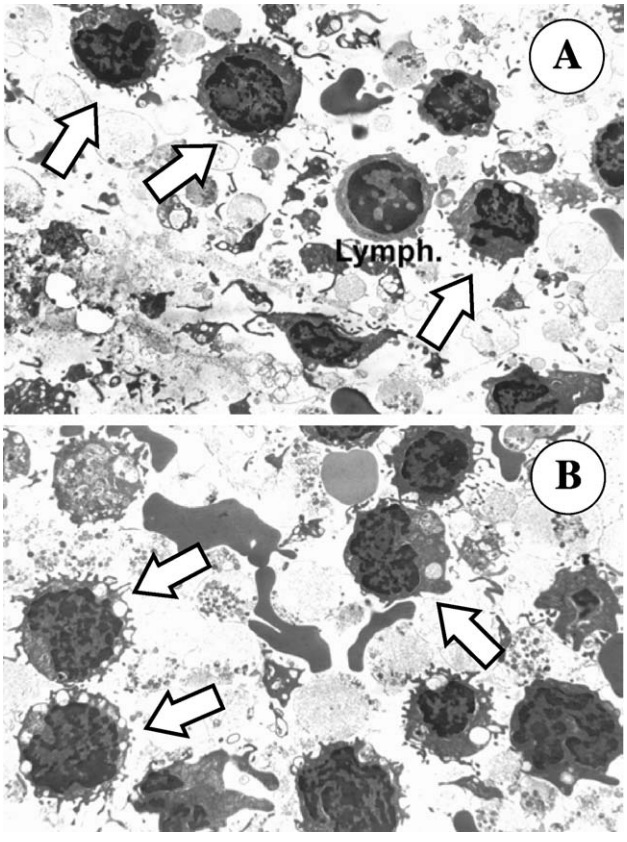

Fig. 2. Representative sample of basophil-rich leukocytes. Electron micrograph of unstimulated (Panel A) and anti-IgE antibodies (1 μg/ml)-stimulated (Panel B) basophils (arrows).

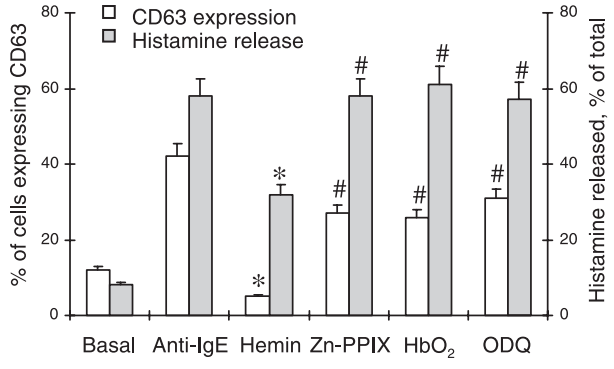

Fig. 3. Hemin (100 μM) reduced the immunological activation and the release of histamine from human basophils stimulated with anti-IgE antibodies (1 μg/ml). This effect was reversed by preincubating the cells for 1 h with Zn-protoporphyrin IX (100 μM), HbO_2 (100 μM) and ODQ (100 μM). The values are expressed as means ± S.E.M. of six experiments. Significance of difference: *$P < 0.01$ vs. anti-IgE, #$p < 0.01$ vs. hemin.

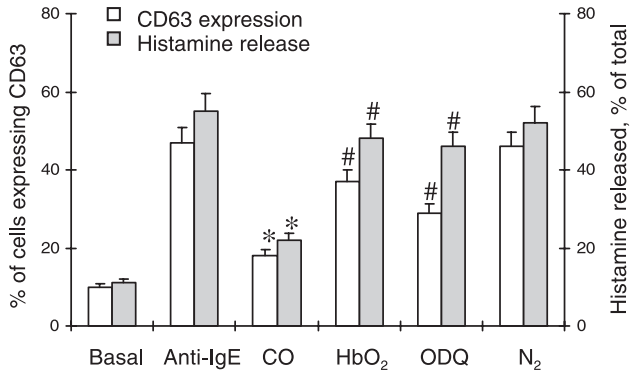

Fig. 4. Carbon monoxide (10 μM for 30 min) reduced the immunological activation and the release of histamine from human basophils stimulated with anti-IgE antibodies (1 μg/ml). This effect was reversed by adding HbO_2 (100 μM) and ODQ (100 μM) for 1 h. N_2 was otherwise uneffective. The values are expressed as means ± S.E.M. of six experiments. Significance of difference: $*P<0.01$ vs. anti-IgE, $\#P<0.01$ vs. CO.

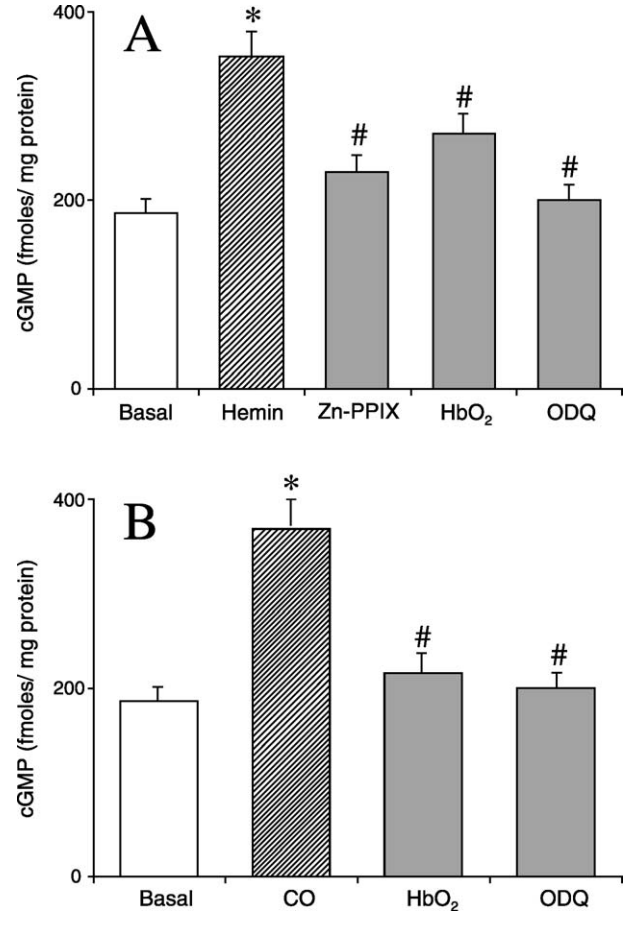

Fig. 5. Hemin (Panel A) and carbon monoxide (Panel B) increased cGMP levels in unstimulated human basophils. This effect was reversed by HbO_2 (100 μM for 1 h) and ODQ (100 μM for 1 h), as well as Zn-protoporphyrin IX (100 μM for 1 h) in the case of hemin. The values are expressed as means ± S.E.M. of six experiments. Significance of difference: $*P<0.01$ vs. basal value, $\#P<0.01$ vs. hemin- and CO-treated cells.

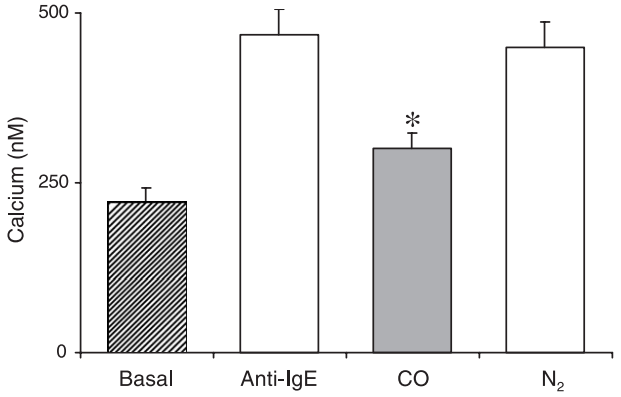

Fig. 6. Carbon monoxide (10 μM for 30 min) decreased $[Ca^{2+}]_i$ levels in human basophils stimulated with anti-IgE antibodies (1 μg/ml). N_2 was otherwise uneffective. The values are expressed as means ± S.E.M. of four experiments. Significance of difference: $*P<0.01$ vs. anti-IgE.

In fact, the expression of the membrane protein CD63, as well as the release of histamine, was significantly reduced in cells exposed to CO, whereas values similar to the controls were obtained in the presence of HbO_2 or ODQ (Fig. 4). The relative hypoxic conditions do not explain the inhibition of basophil immune response, because cells incubated in N_2-saturated buffer fully retained their immunological responsiveness and reacted to anti-IgE by increasing the expression of membrane protein CD63 and by releasing histamine, as did the control cells (Fig. 4).

The activity of heme oxygenase 1 has been reported to regulate cGMP levels in several cell lines through its product, CO (Kharitonov et al., 1995). In our experiments, the basal cGMP levels of enriched human basophils increased markedly in cells preincubated with the heme oxygenase 1 inducer, hemin (100 μM). The hemin-induced increase in cGMP levels was significantly reduced by a 30-min preincubation with Zn-protoporphyrin IX (100 μM), HbO_2 (100 μM) and ODQ (100 μM) (Fig. 5, Panel A). Exogenously administered CO behaved similarly to hemin, for it significantly increased cGMP levels. This effect was blunted by HbO_2 and by ODQ (Fig. 5, Panel B).

As expected, challenge with anti-IgE of enriched human basophils incubated in buffer exposed to air produced a marked increase in cytosolic Ca^{2+} levels; the same results were obtained with cells incubated in N_2-saturated buffer. In human basophils exposed for 30 min to 10 μM CO, the anti-IgE-induced increase in cytosolic Ca^{2+} concentration was significantly reduced (Fig. 6).

4. Discussion

The present experiments show that treatment with hemin, a heme oxygenase 1 substrate, or exposure to exogenous CO reduces the anti-IgE-induced expression of the membrane protein CD63 and decreases the $Fc_\varepsilon RI$-dependent release of histamine from enriched human basophils. This

phenomenon was coupled with an increase in cGMP levels and a decrease in cytosolic Ca^{2+} concentrations in response to anti-IgE. The inhibition of the reported activation of human basophils was prevented by blocking heme oxygenase activity with Zn-protoporphyrin IX, and was reversed by combining CO with HbO_2, or blocking its physiological target, soluble guanylyl cyclase, with ODQ. Nitrogen does not modify the reactivity of human basophils, thus excluding that the relative hypoxia, a condition which stimulates heme oxygenase 1 expression (Morita et al., 1995; Siow et al., 1999), was involved substantially in the observed inhibition.

It is unlikely that hemin acted through a chromoglycate-like membrane-stabilizing effect, since the inhibitory action of hemin was reversed by Zn-protoporphyrin IX, HbO_2 and ODQ, which should be ineffective against chromoglycate-like inhibition. It is also unlikely that the inhibition of the basophil activation afforded by hemin was due to the increased generation of bilirubin, one of the end-products of the heme oxygenase pathway. That bilirubin is an effective antioxidant is well accepted (Minetti et al., 1998), and a protective role for bilirubin against immunological inflammation can be envisaged. However, the full reversal of the inhibition of basophil histamine release and membrane protein CD63 expression afforded by Zn-protoporphyrin IX, by HbO_2 and ODQ indicates that endogenously produced CO is responsible for the inhibition of the immunological activation of human basophils induced by preincubation with hemin.

The present experiments also show that hemin-treated basophils generated bilirubin in substantially higher amounts than did the control cells. Since bilirubin and CO are the end-products of heme oxygenase 1 enzymatic activity, human basophils treated with hemin produced higher amounts of CO than did control cells, and the increased generation of endogenous CO could account for the inhibition of the immunological activation of basophils. This hypothesis is strengthened by the finding that exogenous CO mimicked the inhibitory effect of hemin, the inhibition being reversed by HbO_2 and ODQ.

Both hemin and CO increased cGMP levels in human basophils. Activation of soluble guanylyl cyclase by CO and the consequent increase in cGMP levels have been repeatedly described in other cell types and organs, including human platelets (Brune and Ullrich, 1987; Utz and Ullrich, 1991; Christodoulides et al., 1995; Sammut et al., 1998), vascular smooth muscle cells (Morita et al., 1995) and rat brain (Laitinen et al., 1997). The basis for guanylyl cyclase activation by CO has been provided by Kharitonov et al. (1995), and the cGMP induced by CO is associated with inhibition of platelet aggregation (Brune and Ullrich, 1987), with relaxation of ileal smooth muscle (Utz and Ullrich, 1991), with vasodilatation of various vascular sites (Sammut et al., 1998; Suematsu et al., 1995), and with neurotransmitter release (Laitinen et al., 1997). The idea that a soluble guanylyl cyclase/cGMP system is the main target of CO has been criticized by Coceani et al. (1996), who suggested that the effect of CO involves the inhibition of cytochrome *P*-450 and the associated formation of endothelin-1. However, Hussain et al. (1997) provided further evidence that CO-induced effects are due to activation of soluble guanylyl cyclase by showing that a rather specific inhibitor of soluble guanylyl cyclase, ODQ, directly inhibits the relaxation of rabbit aortic rings induced by CO.

The mechanism by which CO activates soluble guanylyl cyclase is similar to that of NO: both gaseous monoxides bind to heme-iron and induce conformational changes in the catalytic site (Marks et al., 1991). The functional links between NO and CO have been determined, both in the brain and in the cardiovascular system (Siow et al., 1999; Sammut et al., 1998). The interplay between the two gaseous mediators is expressed by the same inhibitory pathway: inhibition of basophil degranulation and histamine release by NO donors (Iikura et al., 1998) as well as by exogenous CO and by a heme oxygenase 1 inducer (this paper).

These findings could have important pharmacological and clinical implications. Basophil-derived CO diffuses to neighboring basophils, where activation of soluble guanylyl cyclase results in elevated intracellular cGMP levels, leading to a decrease in their immunological response in an autocrine/paracrine fashion. Basophil-derived CO may also diffuse to adjacent vascular smooth muscle cells, leading to their relaxation through a similar soluble guanylyl cyclase/cGMP-mediated effect. In addition to activating soluble guanylyl cyclase, the inhibitory actions of CO on basophil activation could be mediated by a direct modulation of cytosolic Ca^{2+} levels.

Carbon monoxide augments the outward K^+ current through Ca^{2+}-dependent potassium channels, thus producing hyperpolarization and decreased Ca^{2+} channel activation. This would maintain cytosolic Ca^{2+} at low levels, unsuitable for triggering the exocytosis of granules typical of anaphylactic degranulation (Dvorak et al., 1996). A similar mechanism has been proposed to explain the soluble guanylyl cyclase-independent vasodilator actions of CO (Wang and Wu, 1997) and the inhibition of carotid body sensory activity by endogenous CO (Prabhakar, 1998). Recent data also suggest that some of the anti-inflammatory effects of carbon monoxide can be due to the activation of the mitogen-activated protein (MAP) kinase signaling pathway, in particular the MAP kinase kinase 3 (MKK 3) /p38 one (Otterbein et al., 2000). The present results, by showing that CO significantly reduces the anti-IgE-induced increase in intracellular Ca^{2+}, could fit with this hypothesis.

Accumulating evidence indicates that the expression of heme oxygenase 1 in vascular endothelial and smooth muscle cells is induced by a variety of agents, including oxidative stress, pro-inflammatory cytokines such as tumor necrosis factor (Wagener et al., 1997), interferon-γ (Motterlini et al., 1998), interleukin-1α (Yet et al., 1997), and peroxynitrite (Foresti et al., 1997), all of which are known to be capable of

inducing and/or of cooperating in the pathogenesis of allergic inflammation. The induction of heme oxygenase 1 activity may therefore provide an endogenous defence mechanism against pro-inflammatory stimuli. Conceivably, the hemin-induced increase in heme oxygenase 1 activity could serve a CO-mediated inhibitory feedback for the allergic activation of basophils, which could be relevant in the modulation of basophil-mediated allergic reactions.

In conclusion, our results indicate, for the first time, that heme oxygenase is expressed in human basophils, and raise the possibility that CO may have a protective effect in inflammatory and allergic disease states. The design of drugs able to modulate the endogenous generation of CO (i.e. activators or inhibitors of the heme oxygenase pathway) would be a new approach to the down-regulation of basophil-mediated and mast cell-mediated allergic reactions.

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References

Bani, D., Baronti, R., Vannacci, A., Bigazzi, M., Sacchi, T.B., Mannaioni, P.F., Masini, E., 2002. Inhibitory effects of relaxin on human basophils activated by stimulation of the Fc_{ε}-receptor. The role of nitric oxide. Int. Immunopharmacol. 2, 1195–1204.

Bergendorff, A., Uvnas, B., 1972. Storage of 5-hydroxytryptamine in rat mast cells. Evidence for an ionic binding to carboxyl groups in a granule heparin-protein complex. Acta Physiol. Scand. 84, 320–331.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brune, B., Ullrich, V., 1987. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. Mol. Pharmacol. 32, 497–504.

Chalmers, A.H., 1991. Simple, sensitive measurement of carbon monoxide in plasma. Clin. Chem. 37, 1442–1445.

Christodoulides, N., Durante, W., Kroll, M.H., Schafer, A.I., 1995. Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. Circulation 91, 2306–2309.

Coceani, F., Kelsey, L., Seidlitz, E., 1996. Carbon monoxide-induced relaxation of the ductus arteriosus in the lamb: evidence against the prime role of guanylyl cyclase. Br. J. Pharmacol. 118, 1689–1696.

Di Bello, M.G., Berni, L., Gai, P., Mirabella, C., Ndisang, J.F., Masini, E., Bani Sacchi, T., Mannaioni, P.F., 1998. A regulatory role for carbon monoxide in mast cell function. Inflamm. Res. 47 (Suppl. 1), S7–S8.

Dvorak, A.M., Schroeder, J.T., MacGlashan Jr., D.W., Bryan, K.P., Morgan, E.S., Lichtenstein, L.M., MacDonald, S.M., 1996. Comparative ultrastructural morphology of human basophils stimulated to release histamine by anti-IgE, recombinant IgE-dependent histamine-releasing factor, or monocyte chemotactic protein-1. J. Allergy Clin. Immunol. 98, 355–370.

Foreman, J.C., 1984. Mast cells and basophil leukocytes. In: Dale, M.M., Foreman, J.C. (Eds.), Textbook of Immunopharmacology. Blackwell, Oxford, pp. 49–53.

Foresti, R., Clark, J.E., Green, C.J., Motterlini, R., 1997. Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. J. Biol. Chem. 272, 18411–18417.

Harper, J.F., Brooker, G., 1975. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2-*o*-acetylation by acetic anhydride in aqueous solution. J. Cycl. Nucleotide Res. 1, 207–218.

Hussain, A.S., Marks, G.S., Brien, J.F., Nakatsu, K., 1997. The soluble guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) inhibits relaxation of rabbit aortic rings induced by carbon monoxide, nitric oxide, and glyceryl trinitrate. Can. J. Physiol. Pharm. 75, 1034–1037.

Iikura, M., Takaishi, T., Hirai, K., Yamada, H., Iida, M., Koshino, T., Morita, Y., 1998. Exogenous nitric oxide regulates the degranulation of human basophils and rat peritoneal mast cells. Int. Arch. Allergy Immunol. 115, 129–136.

Kharitonov, V.G., Sharma, V.S., Pilz, R.B., Magde, D., Koesling, D., 1995. Basis of guanylate cyclase activation by carbon monoxide. Proc. Natl. Acad. Sci. U. S. A. 92, 2568–2571.

Knol, E.F., Mul, F.P., Jansen, H., Calafat, J., Roos, D., 1991. Monitoring human basophil activation via membrane protein CD63 monoclonal antibody 435. J. Allergy Clin. Immunol. 88, 328–338.

Kondo, K., Mitchell, J.A., de Nucci, G., Vane, J.R., 1989. Simultaneous measurement of endothelium-derived relaxing factor by bioassay and guanylate cyclase stimulation. Br. J. Pharmacol. 98, 630–636.

Koranteng, R.D., Dearman, R.J., Kimber, I., Coleman, J.W., 2000. Phenotypic variation in mast cell responsiveness to the inhibitory action of nitric oxide. Inflamm. Res. 49, 240–246.

Kremzer, L.T., Wilson, I.B., 1961. A procedure for the determination of histamine. Biochim. Biophys. Acta 50, 364–367.

Laitinen, K.S., Salovaara, K., Severgnini, S., Laitinen, J.T., 1997. Regulation of cyclic GMP levels in the rat frontal cortex in vivo: effects of exogenous carbon monoxide and phosphodiesterase inhibition. Brain Res. 755, 272–278.

Lichtenstein, L.M., Gillespie, E., 1975. The effects of the H_1 and H_2 antihistamines on ‘‘allergic’’ histamine release and its inhibition by histamine. J. Pharmacol. Exp. Ther. 192, 441–450.

Llesuy, S.F., Tomaro, M.L., 1994. Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. Biochim. Biophys. Acta 1223, 9–14.

Maines, M., 1996. Carbon monoxide and nitric oxide homology: differential modulation of heme oxygenases in brain and detection of protein and activity. Methods Enzymol. 268, 473–488.

Marks, G.S., Brien, J.F., Nakatsu, K., McLaughlin, B.E., 1991. Does carbon monoxide have a physiological function? Trends Pharmacol. Sci. 12, 185–188.

Minetti, M., Mallozzi, C., Di Stasi, A.M., Pietraforte, D., 1998. Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. Arch. Biochem. Biophys. 352, 165–174.

Miroli, A.A., James, B.M., Spitz, M., 1986. Single step enrichment of human peripheral blood basophils by Ficoll-Paque centrifugation. J. Immunol. Methods 88, 91–96.

Morita, T., Perrella, M.A., Lee, M.E., Kourembanas, S., 1995. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc. Natl. Acad. Sci. U. S. A. 92, 1475–1479.

Motterlini, R., Gonzales, A., Foresti, R., Clark, J.E., Green, C.J., Winslow, R.M., 1998. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. Circ. Res. 83, 568–577.

Ndisang, J.F., Gai, P., Berni, L., Mirabella, C., Baronti, R., Mannaioni, P.F., Masini, E., 1999. Modulation of the immunological response of guinea pig mast cells by carbon monoxide. Immunopharmacology 43, 65–73.

Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao, L.H., Wysk, M., Davis, R.J., Flavell, R.A., Choi, A.M., 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat. Med. 6, 422–428.

Prabhakar, N.R., 1998. Endogenous carbon monoxide in control of respiration. Respir. Physiol. 114, 57–64.

Sainte-Laudy, J., Sabbah, A., Vallon, C., Guerin, J.C., 1998. Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release. Inflamm. Res. 47, 401–408.

Salvemini, D., de Nucci, R., Gryglewski, R.J., Vane, J.R., 1989. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. Proc. Natl. Acad. Sci. U. S. A. 86, 6328–6332.

Salvemini, D., Masini, E., Pistelli, A., Mannaioni, P.F., Vane, J.R., 1991. Nitric oxide: a regulatory mediator of mast cell reactivity. J. Cardiovasc. Pharmacol. 17, 258–264.

Sammut, I.A., Foresti, R., Clark, J.E., Exon, D.J., Vesely, M.J., Sarathchandra, P., Green, C.J., Motterlini, R., 1998. Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of heme oxygenase-1. Br. J. Pharmacol. 125, 1437–1444.

Shore, P.A., Burkhalter, A., Cohn, V.H., 1959. A method for the fluorimetric assay of histamine in tissues. J. Pharmacol. Exp. Ther. 127, 182–186.

Siow, R.C., Sato, H., Mann, G.E., 1999. Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? Cardiovasc. Res. 41, 385–394.

Suematsu, M., Goda, N., Sano, T., Kashiwagi, S., Egawa, T., Shinoda, Y., Ishimura, Y., 1995. Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. J. Clin. Invest. 96, 2431–2437.

Thompson, H.L., Metcalfe, D.D., Kinet, J.P., 1990. Early expression of high-affinity receptor for immunoglobulin E (Fc_eRI) during differentiation of mouse mast cells and human basophils. J. Clin. Invest. 85, 1227–1233.

Tomlinson, A., Willoughby, A.D., 1999. Inducible enzymes in inflammation: advances, interactions and conflicts. In: Tomlinson, A., Willoughby, A.D. (Eds.), Inducible Enzymes in Inflammatory Response. Birkäuser, Basel, Switzerland, pp. 187–205.

Tsien, R.Y., Pozzan, T., Rink, T.J., 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. 94, 325–334.

Utz, J., Ullrich, V., 1991. Carbon monoxide relaxes ileal smooth muscle through activation of guanylate cyclase. Biochem. Pharmacol. 41, 1195–1201.

VanUffelen, B.E., de Koster, B.M., VanSteveninck, J., Elferink, J.G., 1996. Carbon monoxide enhances human neutrophil migration in a cyclic GMP-dependent way. Biochem. Biophys. Res. Commun. 226, 21–26.

Vasdev, S., Thompson, P., Triggle, C., Fernandez, P., Bolli, P., Ananthanarayanan, V.S., 1988. Fura-2 used as a probe to show elevated intracellular free calcium in platelets of Dahl-sensitive rats fed a high salt diet. Biochem. Biophys. Res. Commun. 154, 380–386.

Wagener, F.A., Feldman, E., de Witte, T., Abraham, N.G., 1997. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin in vascular endothelial cells. Proc. Soc. Exp. Biol. Med. 216, 456–463.

Wang, R., Wu, L., 1997. The chemical modification of K^+/Ca^{2+} channels by carbon monoxide in vascular smooth muscle cells. J. Biol. Chem. 272, 8222–8226.

Yet, S.F., Pellacani, A., Patterson, C., Tan, L., Folta, S.C., Foster, L., Lee, W.S., Hsieh, C.M., Perrella, M.A., 1997. Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. J. Biol. Chem. 272, 4295–4301.