British Journal of Pharmacology (2009), 156, 1279–1286

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Inhibition of anti-IgE mediated human mast cell activation by NO donors is dependent on their NO release kinetics

KH Yip[1], FP Leung[2], Y Huang[2] and HYA Lau[1]

Departments of [1]Pharmacology and [2]Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China

Background and purpose: Although the mast cell is a source of nitric oxide (NO), the effect of NO on human mast cells has not been defined. This study investigated if exogenous NO could affect human mast cell activation.
Experimental approach: Effects of different NO donors on immunoglobulin E (IgE)-dependent activation of human-cultured mast cells (HCMC) derived from precursors in buffy coat were investigated by measuring histamine release. Intracellular NO in HCMC was monitored with confocal microscopy using the fluorescent NO indicator 4-amino-5-methylamino-2′, 7′-difluorofluorescein.
Key results: Diethylamine NONOate (DEA/NO) and MAHMA NONOate (NOC-9), both have rapid NO release rates, only inhibited anti-IgE-induced histamine release when added to HCMC at the time of activation. NO donors with slower NO release kinetics were ineffective even after 30 min incubation. Confocal microscopy revealed that the effectiveness of NO donors was dependent on the availability of adequate NO inside HCMC during activation. The inhibitory action of DEA/NO was diminished by the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl but potentiated by the anti-oxidant, N-acetylcysteine (NAC). Furthermore, co-incubation with NAC allowed previously ineffective NO donors to suppress HCMC activation and thus suggested that NAC could increase the availability of NO from NO donors.
Conclusions and implications: Our results demonstrated that NO was able to modulate human mast cell activation but only when enough NO was present at the time of cell activation. Our findings explain the controversy over the effectiveness of NO on mast cell degranulation and supports the possibility that NO donors could be beneficial for treating allergic inflammation.
British Journal of Pharmacology (2009) **156**, 1279–1286; doi:10.1111/j.1476-5381.2009.00120.x; published online 19 March 2009

Keywords: histamine; human mast cells; inflammation; nitric oxide; nitric oxide donor

Abbreviations: carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl; cGMP, cyclic guanosine monophosphate; DAF-FM, 4-amino-5-methylamino-2′, 7′-difluorofluorescein; DEA/NO, diethylamine NONOate; FHB/HA, full HEPES buffer with human albumin; HCMC, human-cultured mast cell; IgE, immunoglobulin E; L-NMMA, L-N^G-monomethyl arginine; NAC, N-acetylcysteine; NaNP, sodium nitroprusside; NOC-9, MAHMA NONOate; NONOates, diazeniumdiolates; NOR-3, (±)-(E)-Ethy-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide; RNOS, reactive nitric oxide species; ROS, reactive oxygen species; RPMC, rat peritoneal mast cells; sGC, soluble guanylate cyclase; SIN, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine; SOD, superoxide dismutase; SPER/NO, spermine NONOate

Introduction

Mast cells are pivotal in the pathogenesis of inflammation and allergic hypersensitivity through the release of diverse chemical substances which initiate inflammatory processes leading to the pathological consequences of allergic disorders such as anaphylaxis, allergic rhinitis and asthma (Metcalfe *et al.*, 1997). The release of preformed (histamine, tumour necrosis factor-α) and *de novo* synthesized (prostaglandin D_2) mediators from mast cell contributes mainly to the immediate phase of allergic inflammation (Galli, 2000) while the synthesis of various chemokines and cytokines (tumour necrosis factor-α, interleukin-8) following mast cell activation contributes to chronic inflammation (Gordon *et al.*, 1990). Recent studies also demonstrated that mast cells are capable of synthesizing and releasing nitric oxide (NO) (Bidri *et al.*, 2001; Sekar *et al.*, 2005; Yip *et al.*, 2008).

Correspondence: HYA Lau, Department of Pharmacology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China. E-mail: hyalau@cuhk.edu.hk
Received 26 August 2008; revised 15 October 2008; accepted 19 November 2008

Nitric oxide is a small uncharged free radical that diffuses freely in and out of cells and between cellular compartments (Coleman, 2001). Rapid but transient synthesis of NO at low concentrations by constitutive nitric oxide synthase (NOS) induces fast and transient response in target cells through modulation of intracellular cyclic guanosine monophosphate (cGMP) levels. In contrast, sustained synthesis of NO at high concentrations by inducible NOS induces diverse and complex series of cGMP-independent actions mediated by reactive nitric oxide species (RNOS) (Moncada *et al.*, 1991). Increased levels of NO have been detected in exhaled air of asthmatic patients (Fischer *et al.*, 2002) but whether the increased level of NO produces beneficial or deleterious effects in asthma is still a matter of controversy.

The roles of NO released by mast cells are not currently defined but it is possible that it may modulate the activity of adjacent cells or of mast cells themselves. Salvemini *et al.*, (1991) first reported that histamine release from rat peritoneal mast cells (RPMC) in response to compound 48/80 and calcium ionophore was enhanced by removal of endogenous NO synthesis by using the NOS inhibitor, L-N^G-monomethyl arginine (L-NMMA) and was suppressed by exogenously applied NO from the NO donor sodium nitroprusside (NaNP). Subsequent studies with NOS inhibitors further revealed that NO generated by rodent mast cells could reduce histamine release induced by immunoglobulin E (IgE) directed antibodies (anti-IgE) (Eastmond *et al.*, 1997; Deschoolmeester *et al.*, 1999) and other stimuli such as compound 48/80, substance P and nerve growth factor (Brooks *et al.*, 1999). Similarly, studies using NaNP or other NO-donors also demonstrated that exogenously added NO could inhibit IgE-induced mediator release from rodent mast cells (Bidri *et al.*, 1995; Eastmond *et al.*, 1997; Deschoolmeester *et al.*, 1999) and human skin mast cells (van Overveld *et al.*, 1993). However, contradictory results which failed to obtain similar observations with L-NMMA and NaNP have also been reported (Iikura *et al.*, 1998; Lau and Chow, 1999; Peh *et al.* 2001). The discrepancy may be due to variations in experimental conditions such as the strains of rats, cell density as well as the purity of the mast cell preparations used (Coleman, 2002).

In view of the controversy on the effects of NO donors on mast cell activation in rodents and a lack of extensive studies of this topic in human mast cells, the objectives of the current research were to investigate if NO donors could modulate activation of human mast cells and if the actions were related to NO release so that a more complete understanding of the roles of NO on human mast cell activation could be obtained. The $CD34^+$ hematopoietic stem cells derived human-cultured mast cells (HCMC) employed in the current study have been demonstrated to share the same phenotypic and functional characteristics of lung parenchymal and intestine mucosal mast cells which are predominantly the MC_T subtype (Wang *et al.*, 2006a). HCMC are thus a useful model for studying mast cell biology in human and their homogeneity can eliminate any influence from contaminating cells. NO donors with distinct NO release properties and kinetics were chosen for investigating if NO could modulate immunologically induced histamine release from HCMC. In addition, we directly measured intracellular levels of NO in HCMC using the NO sensitive dye 4-amino-5-methylamino-2′, 7′-difluorofluorescein (DAF-FM) and also tested if the presence of antioxidants could increase the potency of NO donors.

Methods

Histamine release assay

Human-cultured mast cells were cultured as described by Wang *et al.* (2006b). At 12th–14th week of culture, HCMC were harvested and incubated with human myeloma IgE (1 $\mu g \cdot mL^{-1}$) for 2 h at 37°C in Iscove's Modified Dulbecco's Medium in a 5% CO_2 incubator. Sensitized HCMC were washed with full HEPES buffer (137 $mmol \cdot L^{-1}$ NaCl, 5.56 $mmol \cdot L^{-1}$ glucose, 12 $mmol \cdot L^{-1}$ HEPES, 2.7 $mmol \cdot L^{-1}$ KCl, 0.4 $mmol \cdot L^{-1}$ NaH_2PO_4 and 1 $mmol \cdot L^{-1}$ $CaCl_2$ at pH 7.4) supplemented with 0.03% with human albumin (FHB/HA), and were then resuspended in pre-warmed buffer. Pre-warmed HCMC suspension was aliquoted into polystyrene test tubes ($0.5–1.5 \times 10^4$ cells/tube) and then incubated with a NO donor for 0 min or 30 min before being challenged with anti-IgE for further 30 min at 37°C. In studies investigating the effects of N-acetylcysteine (NAC) and superoxide dismutase (SOD), HCMC were incubated with the antioxidant and the NO donor for 30 min prior to activation with anti-IgE. For studying the effect of NO scavenging, diethylamine NONOate (DEA/NO) was pre-mixed with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) for 5 min before adding to HCMC at the time of anti-IgE challenge. Cells incubated with buffer alone served as control for spontaneous histamine release. Reactions were stopped by addition of ice-cold buffer followed immediately by centrifugation at 180× *g* for 5 min at 4°C. Cell pellets and supernatants were separated by transferring the supernatant in each tube to a new tube. Cell pellets were resuspended with distilled water and 30% perchloric acid was added to both the supernatants and cell lysates prior to measuring the histamine contents spectrofluorometrically with a Bran+Luebbe AutoAnalyzer 3. Results were expressed as a percentage of total cellular histamine release [Histamine release (%)] or a percentage of the anti-IgE-induced histamine release that was inhibited in the presence of a NO donor [Inhibition (%)]. Histamine release (%) was calculated by the equation $[a/(a + b)] \times 100\%$ where a is the amount of histamine released into the supernatant and b is the amount of histamine remaining in the cell pellet after centrifugation. The spontaneous histamine release levels of HCMC before and after sensitization with myeloma IgE were comparable and were less than 5% of total cellular histamine.

Fluorimetric detection of NO

Intracellular level of NO was monitored using the fluorescent NO indicator DAF-FM diacetate. Mast cells seeded on glass coverslips were incubated with 1 $\mu mol \cdot L^{-1}$ of DAF-FM diacetate in FHB/HA at 37°C for 30 min. Fluorimetric measurements were performed using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc.,

Melville, NY, USA) mounted on an inverted IX81 Olympus microscope, equipped with a 20× water-immersion objective (NA 0.5). Fluorescence intensity excited at 495 nm and emitted at 515 nm was measured for 5 min. Changes in intracellular NO were expressed as F_1/F_0 ratios where F_1 and F_0 was the fluorescence intensity at a specific time and at the initiation of image recording.

Materials

NaNP, S-nitroso-N-acetylpenicillamine (SNAP), 3-Morpholinosydnonimine (SIN-1), (±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (NOR-3), Diethylamine NONOate (DEA/NO), MAHMA NONOate (NOC-9), and Spermine NONOate (SPER/NO), human myeloma IgE, superoxide dismutase (SOD) were purchased from Calbiochem (San Diego, CA). NAC and goat anti-human immunoglobulin E antibody (epsilon-chain specific) were from Sigma (St. Louis, MO). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (Carboxy-PTIO) was from Merck (Darmstadt, Germany) and 4-amino-5-methylamino-2′, 7′-difluorofluorescein (DAF-FM) diacetate was from Invitrogen (Carlsbad, CA).

Statistical analysis

All values are expressed as mean ± standard error of the mean. Significance of the mean differences in each experiment was analysed using Student's *t*-test while the differences between treatment groups were analysed with two-way ANOVA.

Result

Effects of NO donors on anti-IgE-induced histamine release in HCMC

Nitric oxide donors were added to IgE-sensitized HCMC at the time of or 30 min before immunological challenge with anti-IgE. None of the NO donors affected the spontaneous level of histamine release (4.9 ± 0.7%) even after 60 min of incubation with HCMC alone. When added simultaneously with anti-IgE to HCMC, DEA/NO and MAHMA NONOate (NOC-9) produced significant and dose-dependent inhibition of anti-IgE-induced histamine release whereas NaNP, S-nitroso-N-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), (±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (NOR-3) and spermine NONOate (SPER/NO) all failed to demonstrate significant effects (Figure 1A). The inhibitory actions of DEA/NO and NOC-9 were reduced to non-significant levels when these NO donors were pre-incubated with HCMC for 30 min prior to anti-IgE challenge while the other NO donors remained ineffective (Figure 1B).

Further investigations revealed that the inhibitory actions of DEA/NO and NOC-9 were diminished even after 10 min pre-incubation with HCMC (Figure 2). At 10^{-4} mol·L^{-1}, DEA/NO clearly inhibited anti-IgE-induced histamine release when added to HCMC at the time of activation but this inhibition was halved when DEA/NO was pre-incubated for 10 min ($P < 0.05$) and, after 30 min pre-incubation, no inhibition was observed. As for 10^{-4} mol·L^{-1} NOC-9, the level of

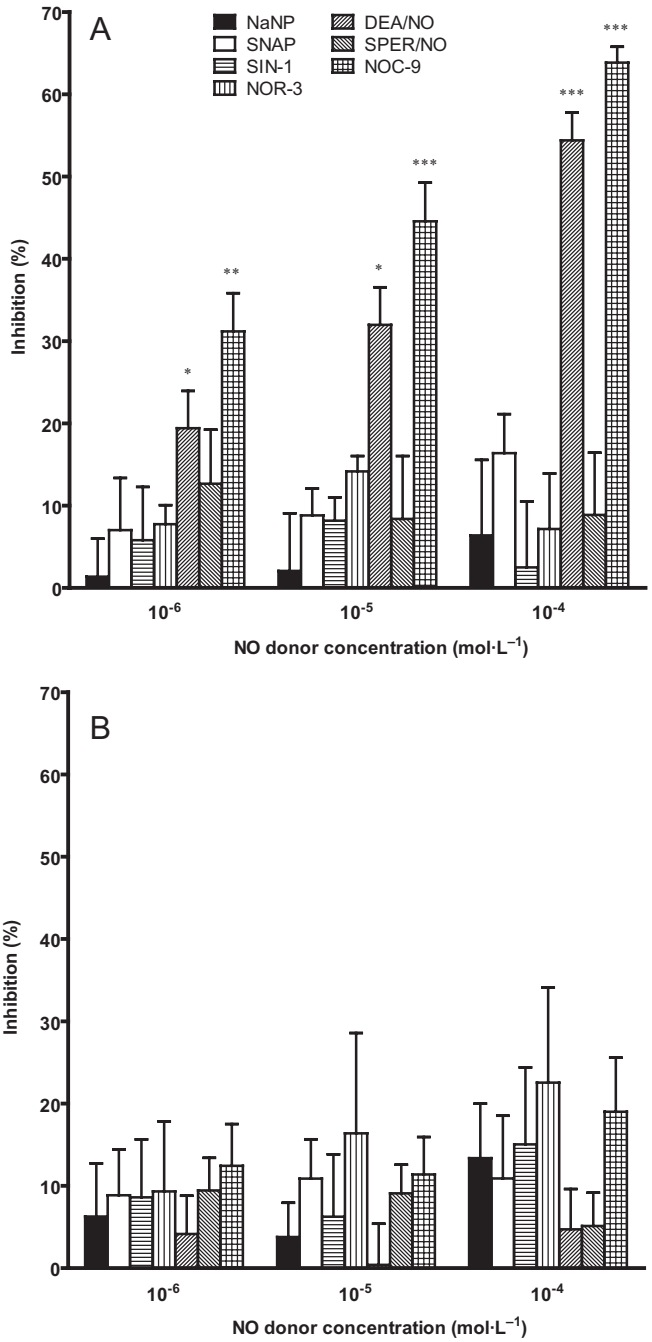

Figure 1 Effects of NO donors on anti-IgE-induced histamine release from HCMC. Sensitized HCMC were incubated with different NO donors for (A) 0 min and (B) 30 min and then challenged with anti-IgE for 30 min to induce histamine release. The levels of anti-IgE-induced histamine release were between 41 ± 3 to 55 ± 3% for 0 min and 40 ± 5 to 48 ± 4% for 30 min pre-incubation. Results were expressed as the percentage of anti-IgE-induced histamine release that was inhibited in the presence of a NO donor [Inhibition (%)]. All values are mean ± SEM of three to six experiments. *$P < 0.05$ when the histamine release induced by anti-IgE alone was significantly reduced in the presence of the NO donor after comparing the means of the actual histamine release levels with Student's *t*-test. DEA/NO, diethylamine NONOate; HCMC, human cultured mast cell; IgE, immunoglobulin E; NaNP, sodium nitroprusside; NO, nitric oxide; NOC-9, MAHMA NONOate; NONOates, diazeniumdiolates; NOR-3, (±)-(E)-Ethyl-2- [(E)-hydroxyimino]-5-nitro-3-hexeneamide; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine; SPER/NO, spermine NONOate.

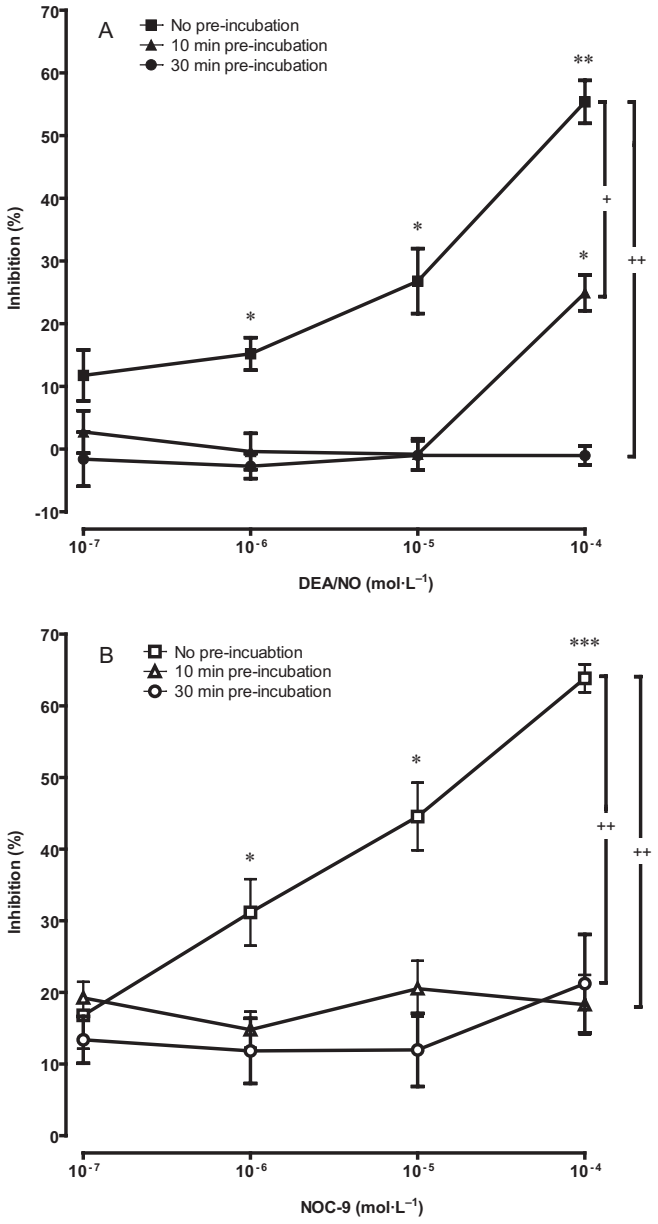

Figure 2 Time course experiments on inhibitory effects of DEA/NO and NOC-9. Sensitized HCMC were pre-incubated with (A) DEA/NO or (B) NOC-9 for 0 min, 10 min or 30 min before challenge with anti-IgE for 30 min to induce histamine release. Anti-IgE induced 52 ± 2%, 54 ± 2% and 53 ± 2% of histamine release respectively for 0 min, 10 min and 30 min pre-incubation. Results were expressed as the percentage of anti-IgE-induced histamine release that was inhibited in the presence of a NO donor [Inhibition (%)]. All values are mean ± SEM of three experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for indicating significant difference between the level of histamine release induced by anti-IgE alone and that in the presence of the NO donor after comparing the means of the actual histamine release levels with Student's *t*-test. +$P < 0.05$, ++$P < 0.01$, for indicating significant difference between the overall level of inhibition of anti-IgE-induced histamine release after pre-incubation and that of no pre-incubation with the NO donor using two-way ANOVA. DEA/NO, diethylamine NONOate; HCMC, human cultured mast cell; IgE, immunoglobulin E; NO, nitric oxide; NOC-9, MAHMA NONOate; NONOates, diazeniumdiolates.

anti-IgE-induced histamine release was significantly inhibited only when the NO donor was added at the time of activation and there was no effect on histamine release following 10 min and 30 min pre-incubation.

Correlation of NO release with the effects of NO donors on HCMC

As the suppression of HCMC activation was only observed with DEA/NO and NOC-9 but not demonstrated by the other NO donors, we investigated if the difference was due to the different level of NO released from these compounds at the time of HCMC activation. SNAP, a commonly used NO donor, was selected to compare with DEA/NO for their capabilities in elevating intracellular NO. Confocal microscopy using the NO sensitive DAF-FM diacetate detected that intracellar NO in HCMC started to increase at 100 s after addition of 10^{-4} mol·L^{-1} DEA/NO but no significant change in base-line fluorescence was observed in cells incubated with SNAP even after 5 min (Figure 3A).

In order to further confirm that the action of DEA/NO was related to its ability to release NO, the NO donor was pre-mixed with the NO scavenger, carboxy-PTIO, before addition to HCMC. Carboxy-PTIO alone produced no significant effect on HCMC activation (<10%) and had no effect on the basal fluorescence in DAF-FM loaded HCMC (Figure 3B,C). Functional study of histamine release indicated that inhibition by 10^{-4} mol·L^{-1} DEA/NO was markedly reduced ($P < 0.01$) in the presence of 10^{-6} mol·L^{-1} carboxyl-PTIO (Figure 3B), while the increase in intracellular NO signal following incubation with DEA/NO was also totally abolished (Figure 3C).

Effects of antioxidants, NAC and SOD, on the actions of NO donors on HCMC

In order to investigate if the lack of mast cell inhibitory effect in SNAP and NaNP and if the loss of such action in DEA/NO after 30 min pre-incubation with HCMC before anti-IgE challenge were due to the rapid degradation of released NO, we studied the effects of antioxidants which had been reported to stabilize NO (Iikura *et al*., 1998; Salvemini *et al*., 1991). NAC, which stabilizes NO in bioactive forms, and SOD, which protects NO from superoxide attack, are used in the current study. In the presence of 5 mmol·L^{-1} NAC, the NO donors (SNAP, NaNP and DEA/NO) could significantly and dose-dependently suppress activation of HCMC even after 30 min pre-incubation (Figure 4). In contrast to NAC, the inhibitory effect of NO donors could not be restored by the presence of 60 U and 100 U SOD (Figure 5A–C).

Discussion

Nitric oxide donors have been widely used as surrogate sources of the gaseous autacoid. While many biological actions of NO donors are directly related to the cellular actions of NO mediated through soluble guanylate cyclase (sGC), there are also frequently reported contradictory *in vitro* biological actions due to differences in the NO releasing mechanisms and the rate of NO release among the different

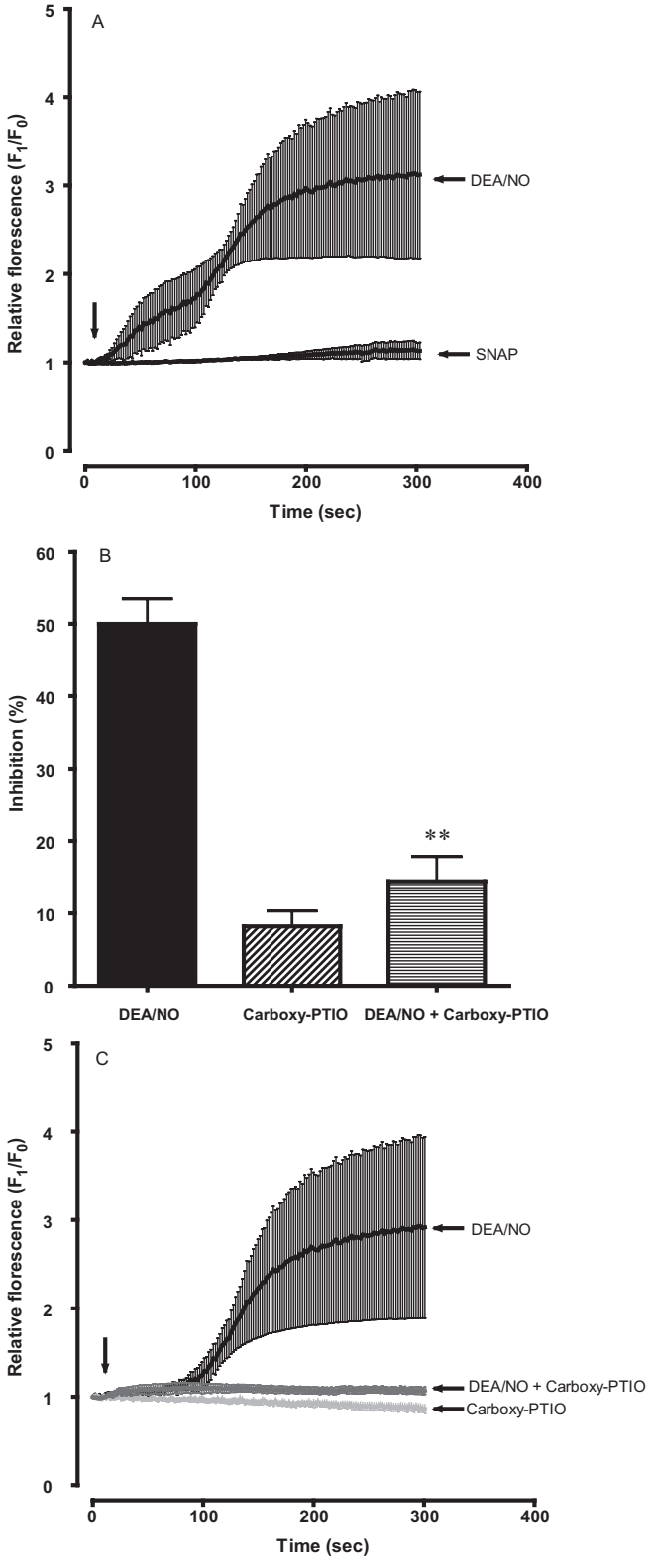

Figure 3 Correlation of NO release with the effects of NO donors on HCMC. (A) Detection of intracellular NO level following incubation with DEA/NO and SNAP. Intracellular NO was monitored in DAF-FM diacetate-fluorescent dye loaded HCMC. Changes in intracellular NO were monitored at 2 s intervals for 5 min and expressed as F_1/F_0 ratios where F_1 and F_0 was the fluorescence intensity at a specific time and at the initiation of image recording respectively. The data presented are mean ± SEM of four experiments. (B) Effect of carboxy-PTIO on the inhibitory action of DEA/NO on HCMC histamine release. Carboxy-PTIO (10^{-6} mol·L^{-1}) was pre-mixed with 10^{-4} mol·L^{-1} DEA/NO before addition to sensitized HCMC and challenged with anti-IgE for 30 min for histamine release. Anti-IgE induced the release of 50 ± 4% of total cellular histamine. Results were expressed as the percentage of anti-IgE-induced histamine release that was inhibited in the presence of a NO donor [Inhibition (%)]. All values are mean ± SEM of five experiments. $^{**}P < 0.01$ indicates that the level of DEA/NO-induced inhibition is significantly different from that in the presence of carboxy-PTIO using Student's *t*-test. (C) Effect of carboxy-PTIO (10^{-6} mol·L^{-1}) on the DEA/NO-induced intracellular NO increase. Changes in intracellular NO were monitored in DAF-FM diacetate-fluorescent dye loaded HCMC at 2s intervals for 5 min and expressed as F_1/F_0 ratios where F_0 was the fluorescence intensity of the initial image during the recording. The data presented are mean ± SEM of four experiments. Carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3- oxide-1-oxyl; DAF-FM, 4-amino-5-methylamino-2′, 7′-difluorofluorescein; DEA/NO, diethylamine NONOate; HCMC, human cultured mast cell; IgE, immunoglobulin E; NO, nitric oxide; NONOates, diazeniumdiolates; SNAP, S-nitroso-N-acetylpenicillamine.

classes of NO donors (Napoli and Ignarro, 2003). Moreover, inconsistencies in the suppressive actions of NO donors on mast cells have been suggested to be due to variations in the degrees of purity and phenotypes of mast cells (Coleman, 2002). Our current study employed highly homogeneous populations of HCMC which resembled MC_T mast cells of human lungs and thus directly confirmed that NO donors are capable of inhibiting anti-IgE-induced activation of human mast cells under appropriate conditions. Furthermore, we have provided evidence suggesting that the failure of some earlier reports in observing the inhibitory action of NO donors on mast cells could be related to the availability of NO at the time of mast cell activation.

As the mast cell inhibitory effects of DEA/NO and NOC-9 reduced as pre-incubation time was increased, the availability of NO at the time of mast cell activation became an important factor to be investigated. Both effective NO donors belong to the diazeniumdiolates (NONOates) group which differs from other chemical classes of NO donors by releasing NO spontaneously and is independent of biological agents at physiological pH with first-order kinetic (Maragos *et al.*, 1991). Among this group of NO donors, DEA/NO and NOC-9 exhibit rapid NO release kinetics at physiological conditions with NO release half-life ($t_{1/2}$) of 2.1 and 1.12 min respectively while SPER/NO has a much slower rate of release ($t_{1/2}$ = 39 min) (Maragos *et al.*, 1991). NO release profiles, measured electrochemically, revealed that DEA/NO demonstrated a quick, high and short burst NO release, while SPER/NO resulted in a slow, low but sustained NO liberation (Thomas *et al.*, 2002). The failure of the slowly NO releasing SPER/NO to demonstrate the same actions of DEA/NO and NOC-9 on HCMC suggested that the rate of NO release is crucial to the mast cell inhibitory actions of NO donors. Similar correlation between the rates of NO release to their vasorelaxing actions among different NONOates members had also been reported (Di Fulvio *et al.*, 2003).

Using a mathematical model, Kavdia and Lewis (2003) demonstrated that a fast releasing NO donor distributed NO more

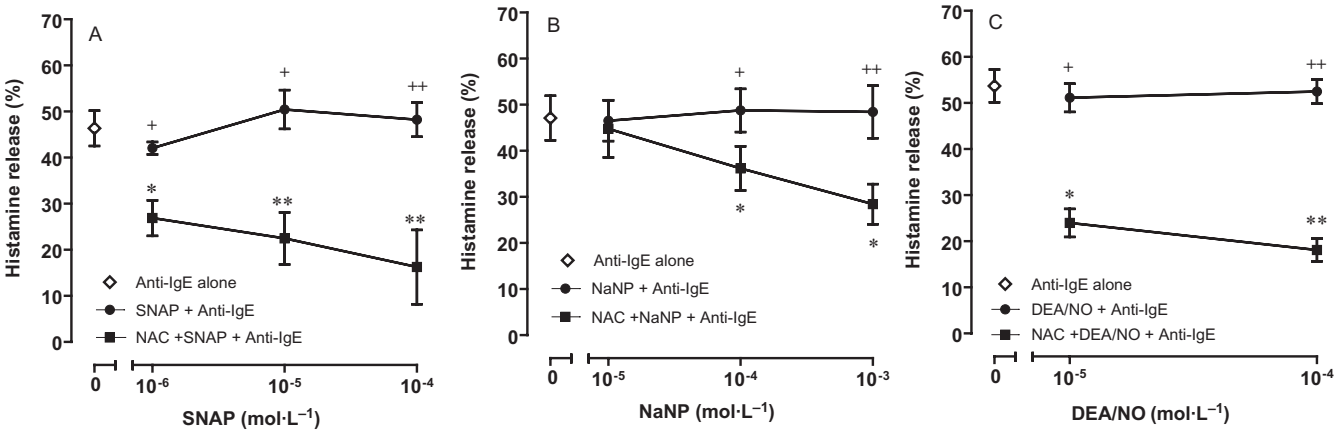

Figure 4 The combined effect of NAC and NO donors on anti-IgE-induced histamine release from HCMC. Sensitized HCMC were first incubated with 5 mmol·L^{-1} NAC and (A) SNAP, (B) NaNP or (C) DEA/NO for 30 min and then challenged with anti-IgE for further 30 min for histamine release. Results were the percentage of total cellular histamine released after corrections for spontaneous release of not more than 5%. All values are mean ± SEM of four to five experiments. Data were analysed with Student's *t*-test and significant differences between histamine release induced by anti-IgE in NO donors with or without NAC are indicated by crosses: $+P < 0.05$, $++P < 0.01$ while those between histamine release induced by anti-IgE alone and in the presence of NO donors and NAC are indicated by asterisks: $*P < 0.05$, $**P < 0.01$. DEA/NO, diethylamine NONOate; HCMC, human cultured mast cell; IgE, immunoglobulin E; NAC, N-acetylcysteine; NaNP, sodium nitroprusside; NO, nitric oxide; NONOates, diazeniumdiolates; SNAP, S-nitroso-N-acetylpenicillamine.

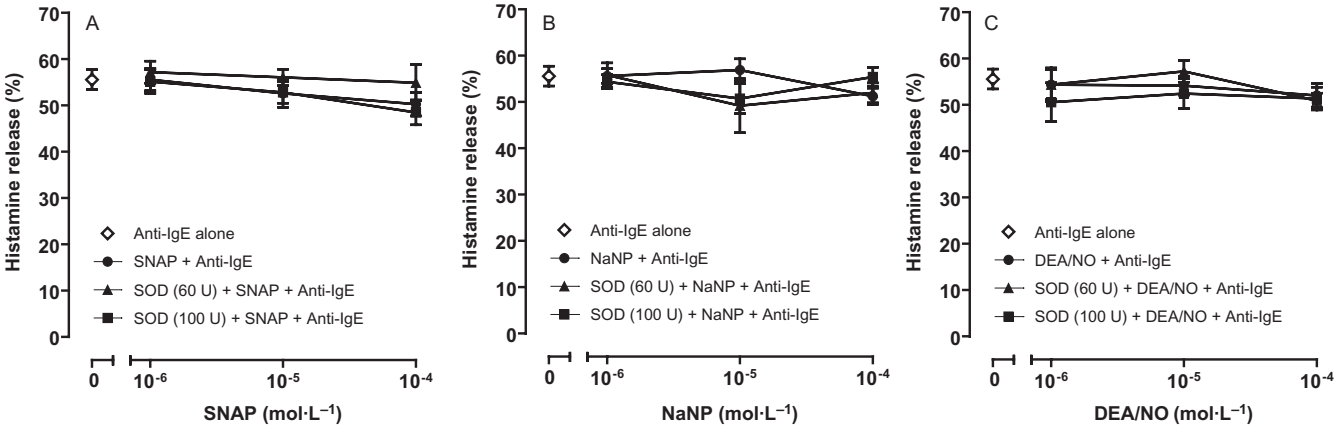

Figure 5 The combined effect of SOD and NO donors on anti-IgE-induced histamine release from HCMC. Sensitized HCMC were first incubated with SOD (60 U or 100 U) and (A) SNAP, (B) NaNP or (C) DEA/NO for 30 min and then challenged with anti-IgE for further histamine release. Results were the percentage of total cellular histamine released after corrections for spontaneous release of not more than 5%. All values are mean ± SEM of three experiments. DEA/NO, diethylamine NONOate; HCMC, human cultured mast cell; IgE, immunoglobulin E; NaNP, sodium nitroprusside; NO, nitric oxide; NONOates, diazeniumdiolates; SNAP, S-nitroso-N-acetylpenicillamine; SOD, superoxide dismutase.

evenly in stagnant media at a given time than a slow releasing NO donor. They proposed that the kinetics of NO release was in general more important than the total NO released by an NO donor as the kinetics affected the spatial and temporal NO exposure levels to cells. Apart from the NONOates, the rates of NO release by other classes of NO donors are difficult to predict as their NO release mechanisms are further complicated by biotransformations facilitated by enzymes or other cofactors or both (Napoli and Ignarro, 2003). In accordance with previous studies using chemiluminescence (Morley *et al.*, 1993) or electrochemical detection (Wink *et al.*, 1996) which showed that DEA/NO produced an immediate and high NO output as compared with NaNP and SNAP, a significant increase of fluorescence signal was detected in DAF-FM loaded HCMC after incubation with DEA/NO for 100 s in the current study but not SNAP. The observation provided direct evidence for NO released from DEA/NO entering HCMC immediately. The importance of this intracellular NO increase in the control of mast cell activation was confirmed by the suppression of the DEA/NO-induced mast cell inhibition and increased intracellular NO, by the specific NO scavenger, carboxy-PTIO.

As inhibition of anti-IgE-induced histamine release was observed only when significant amounts of NO were present at the time of mast cell activation, we hypothesized that the failure of most NO donors to demonstrate inhibitory action on mast cells was due to the rapid degradation of NO released by the donors. NO has a short half life and decomposes into other nitrogen oxides (NO_2^- and NO_3^-). NO availability in biological systems is further decreased by reactive oxygen species (ROS) (Steiner *et al.*, 2002; Swindle and Metcalfe, 2007) which rapidly react with NO to form RNOS, such as

$ONOO^-$ (Swindle and Metcalfe, 2007). By reducing the inactivation of NO, NAC and SOD potentiate the actions of NO donors in many systems, such as anti-aggregation of human platelets (Anfossi *et al.*, 2001), attenuation of ischemia-reperfusion injury in rat kidney (Dobashi *et al.*, 2002), cardioprotection of ischaemic attack of guinea pig heart (Masini *et al.*, 1999) as well as inhibition of histamine release in RPMC (Iikura *et al.*, 1998). Although NAC (2.5–10 $mmol \cdot L^{-1}$) alone was reported to inhibit anti-IgE-mediated exocytosis in RBL-2H3 cells by inhibition of Syk kinase (Valle and Kinet, 1995), NAC at 5 $mmol \cdot L^{-1}$ alone did not modulate HCMC activation in our current study (result not shown). Instead, our results agreed with a previous report by Iikura *et al.* (1998) on RPMC, that NAC augmented the ability of NO donors to suppress immunologically induced histamine release. In contrast, SOD was not effective in supporting the inhibitory actions of NO donors on HCMC. As SOD reduces NO inactivation primarily by interfering with the formation of ROS, the ineffectiveness of SOD thus suggests that ROS are not a factor for the failure of NO donors to suppress mast cell activation. NAC is believed to prolong the action of NO by enhancing the bioconversion of nitrates to NO and by forming stable S-nitrosothiols. It has been reported that in the plasma, NO can easily react with albumin to form high molecular weight S-nitrosothiols (S-NO-albumin), which release NO directly and serve as a buffer and reservoir of relatively stable NO (Scharfstein *et al.*, 1994). NAC, being a low molecular weight thiol, can compete for NO with albumin to form low molecular weight S-nitrosothiols by transnitrosation rapidly and completely *in vivo* under physiological conditions (Bisseling *et al.*, 2004). These smaller, less diffusion-limited NO adducts can then contribute to a progressive release of NO which enhances the beneficial roles of endogenously produced NO in the vasculature (Stamler *et al.*, 1992). NAC has been demonstrated to interact with NO to form a NO donor (S-nitroso-N-cysteine) which acts as an sGC activator and such interaction was implicated in the modulation of platelet function by NAC (Anfossi *et al.*, 2001). Accordingly, the reduced conversion of NO to nitrogen oxides and the formation of low molecular weight S-nitrosothiols together allow substantial level of functional NO adducts to accumulate over 30 min which then interfere with the activation of HCMC in a similar fashion to the immediate elevation of NO level mediated by the rapidly releasing NO donors, when added together with anti-IgE.

Taken together, our current study demonstrates that the effectiveness of an NO donor in inhibiting mast cell activation is closely related to the rate of NO release from the donor. NO donors with rapid rates of spontaneous NO release increase NO to a level that is critical for HCMC inhibition during anti-IgE activation. Inconsistency in previous studies may be due to the differences in experimental protocols and conditions which affected the NO availability in the system. Furthermore, intrinsic differences in the different mast cell models employed and the diverse mechanisms of actions expressed by different NO donors may also be contributing factors. For instance, in contrary to our observations, the slow release NO donor, S-nitrosoglutathione (SNOG), was reported to significantly inhibit leukotriene C_4 release from human mast cell-like LAD-2 cells after 30 min pre-incubation (Gilchrist *et al.*, 2004). Apart from the intrinsic differences between the two human mast cell models, the discrepancy between these two studies may be due to the different mechanisms of actions of the NO donors employed. Our results are in line with the suggestion of Gordge *et al.* (1998) that the close relationship between NO release kinetics of NO donors and their biological actions, such as inhibition of platelet aggregation in this case, would indicate a cGMP-dependent mechanism. These authors, however, observed in the same study that the platelet inhibitory potency of SNOG did not correlate with its slow rate of NO release and further demonstrated that SNOG was capable of activating a cGMP-independent mechanism that involved both copper (I) ions and surface membrane thiol groups. Inhibition of LAD-2 cells by SNOG is thus possibly through this alternative mechanism. Further studies obviously are required to confirm our speculation. The current study further provides a better understanding of how NO can produce beneficial effects in the pathogenesis of inflammatory responses through the inhibition of mast cells. Moreover, we demonstrated that low molecular weight thiol containing compounds such as NAC could be used to facilitate the anti-inflammatory actions of NO and thus could be used for developing effective anti-inflammatory molecules for the clinical management of inflammatory diseases such as asthma.

Acknowledgement

This work was fully supported by the Research Grants Council of Hong Kong (4337/03M).

Conflicts of interest

None to declare.

References

Anfossi G, Russo I, Massucco P, Mattiello L, Cavalot F, Trovati M (2001). N-acetyl-L-cysteine exerts direct anti-aggregating effect on human platelets. *Eur J Clin Invest* **31**: 452–461.

Bidri M, Becherel PA, Le Goff L, Pieroni L, Guillosson JJ, Debre P *et al.* (1995). Involvement of cyclic nucleotides in the immunomodulatory effects of nitric oxide on murine mast cells. *Biochem Biophys Res Commun* **210**: 507–517.

Bidri M, Feger F, Varadaradjalou S, Ben Hamouda N, Guillosson JJ, Arock M (2001). Mast cells as a source and target for nitric oxide. *Int Immunopharmacol* **1**: 1543–1558.

Bisseling TM, Maria Roes E, Raijmakers MT, Steegers EA, Peters WH, Smits P (2004). N-acetylcysteine restores nitric oxide-mediated effects in the fetoplacental circulation of preeclamptic patients. *Am J Obstet Gynecol* **191**: 328–333.

Brooks AC, Whelan CJ, Purcell WM (1999). Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition. *Br J Pharmacol* **128**: 585–590.

Coleman JW (2001). Nitric oxide in immunity and inflammation. *Int Immunopharmacol* **1**: 1397–1406.

Coleman JW (2002). Nitric oxide: a regulator of mast cell activation and mast cell-mediated inflammation. *Clin Exp Immunol* **129**: 4–10.

Deschoolmeester ML, Eastmond NC, Dearman RJ, Kimber I, Basketter

DA, Coleman JW (1999). Reciprocal effects of interleukin-4 and interferon-gamma on immunoglobulin E-mediated mast cell degranulation: a role for nitric oxide but not peroxynitrite or cyclic guanosine monophosphate. *Immunology* **96**: 138–144.

Di Fulvio M, Lauf PK, Shah S, Adragna NC (2003). NONOates regulate KCl cotransporter-1 and -3 mRNA expression in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* **284**: H1686–92.

Dobashi K, Singh I, Orak JK, Asayama K, Singh AK (2002). Combination therapy of N-acetylcysteine, sodium nitroprusside and phosphoramidon attenuates ischemia-reperfusion injury in rat kidney. *Mol Cell Biochem* **240**: 9–17.

Eastmond NC, Banks EM, Coleman JW (1997). Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J Immunol* **159**: 1444–1450.

Fischer A, Folkerts G, Geppetti P, Groneberg DA (2002). Mediators of asthma: nitric oxide. *Pulm Pharmacol Ther* **15**: 73–81.

Galli SJ (2000). Mast cells and basophils. *Curr Opin Hematol* **7**: 32–39.

Gilchrist M, McCauley SD, Befus AD (2004). Expression, localization, and regulation of NOS in human mast cell lines: effects on leukotriene production. *Blood* **104**: 462–469.

Gordge MP, Hothersall JS, Noronha-Dutra AA (1998). Evidence for a cyclic GMP-independent mechanism in the anti-platelet action of S-nitrosoglutathione. *Br J Pharmacol* **124**: 141–148.

Gordon JR, Burd PR, Galli SJ (1990). Mast cells as a source of multifunctional cytokines. *Immunol Today* **11**: 458–464.

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

Kavdia M, Lewis RS (2003). Nitric oxide delivery in stagnant systems via nitric oxide donors: a mathematical model. *Chem Res Toxicol* **16**: 7–14.

Lau HY, Chow SM (1999). Nitric oxide does not affect histamine release from rat peritoneal mast cells. *Inflamm Res* **48** (Suppl. 1): S27–8.

Maragos CM, Morley D, Wink DA, Dunams TM, Saavedra JE, Hoffman A *et al*. (1991). Complexes of .NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *J Med Chem* **34**: 3242–3247.

Masini E, Salvemini D, Ndisang JF, Gai P, Berni L, Moncini M *et al*. (1999). Cardioprotective activity of endogenous and exogenous nitric oxide on ischaemia reperfusion injury in isolated guinea pig hearts. *Inflamm Res* **48**: 561–568.

Metcalfe DD, Baram D, Mekori YA (1997). Mast cells. *Physiol Rev* **77**: 1033–1079.

Moncada S, Palmer RM, Higgs EA (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43**: 109–142.

Morley D, Maragos CM, Zhang XY, Boignon M, Wink DA, Keefer LK (1993). Mechanism of vascular relaxation induced by the nitric oxide (NO)/nucleophile complexes, a new class of NO-based vasodilators. *J Cardiovasc Pharmacol* **21**: 670–676.

Napoli C, Ignarro LJ (2003). Nitric oxide-releasing drugs. *Annu Rev Pharmacol Toxicol* **43**: 97–123.

Peh KH, Moulson A, Wan BY, Assem EK, Pearce FL (2001). Role of nitric oxide in histamine release from human basophils and rat peritoneal mast cells. *Eur J Pharmacol* **425**: 229–238.

Salvemini D, Masini E, Pistelli A, Mannaioni PF, Vane J (1991). Nitric oxide: a regulatory mediator of mast cell reactivity. *J Cardiovascular Pharm* **17**: S258–S264.

Scharfstein JS, Keaney JF Jr, Slivka A, Welch GN, Vita JA, Stamler JS *et al*. (1994). In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols. *J Clin Invest* **94**: 1432–1439.

Sekar Y, Moon TC, Munoz S, Befus AD (2005). Role of nitric oxide in mast cells: controversies, current knowledge, and future applications. *Immunol Res* **33**: 223–239.

Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J *et al*. (1992). Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci USA* **89**: 7674–7677.

Steiner DR, Gonzalez NC, Wood JG (2002). Interaction between reactive oxygen species and nitric oxide in the microvascular response to systemic hypoxia. *J Appl Physiol* **93**: 1411–1418.

Swindle EJ, Metcalfe DD (2007). The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. *Immunol Rev* **217**: 186–205.

Thomas DD, Miranda KM, Espey MG, Citrin D, Jourd'heuil D, Paolocci N *et al*. (2002). Guide for the use of nitric oxide (NO) donors as probes of the chemistry of NO and related redox species in biological systems. *Methods Enzymol* **359**: 84–105.

Valle A, Kinet JP (1995). N-acetyl-L-cysteine inhibits antigen-mediated Syk, but not Lyn tyrosine kinase activation in mast cells. *FEBS Lett* **357**: 41–44.

van Overveld FJ, Bult H, Vermeire PA, Herman AG (1993). Nitroprusside, a nitrogen oxide generating drug, inhibit release of histamine and tryptase from human skin mast cells. *Agents Actions* Special Conference Issue C237–C238.

Wang XS, Sze Wing S, Kwok Ho Y, Lau HY (2006a). Functional characterization of human mast cells cultured from adult peripheral blood. *Int Immunopharmacol* **6**: 839–847.

Wang XS, Yip KH, Sam SW, Lau HY (2006b). Buffy coat preparation is a convenient source of progenitors for culturing mature human mast cells. *J Immunol Methods* **309**: 69–74.

Wink DA, Cook JA, Pacelli R, DeGraff W, Gamson J, Liebmann J *et al*. (1996). The effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity: a direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys* **331**: 241–248.

Yip KH, Huang Y, Waye MM, Lau HY (2008). Induction of nitric oxide synthases in primary human cultured mast cells by IgE and proinflammatory cytokines. *Int Immunopharmacol* **8**: 764–768.