



PRELIMINARY REPORT

Induction of nitric oxide synthases in primary human cultured mast cells by IgE and proinflammatory cytokines

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Abstract

The mast cells have been suggested to be a cellular source of nitric oxide (NO) which level is increased in the pathogenesis of asthma. However, isoforms of the NO generating enzyme, nitric oxide synthase (NOS), in primary human mast cells have not been defined due to the lack of a suitable model. We hence examined directly the expression of NOS mRNA and proteins in primary human cultured mast cells (HCMC). Mature HCMC were cultured from CD34⁺ progenitors isolated from buffy coat preparations and were subjected to IgE sensitisation, IgE receptor mediated activation and cytokines induced stimulation. While expression of NOS mRNA was detected by conventional reverse transcription-polymerase chain reaction (RT-PCR) and quantitatively analyzed with real-time RT-PCR, expression of NOS proteins was detected by immunostaining. In non-stimulated HCMC incubated in medium alone, expressions of NOS were not detected. While overnight incubation of HCMC with IgE significantly increased the expression of NOS2 and NOS3, only NOS2 expression was up-regulated after overnight incubation with a mixture of TNF- α , IFN- γ and IL-1 β . Cross-linking of IgE with anti-IgE further increased NOS2 expression with a concomitant decrease in NOS3 expression. NOS1 was not detected in all treatments. In conclusion, we have shown for the first time that NOS2 and NOS3 expressions are induced in primary human mast cells following appropriate stimulations. Comparisons between the differential expressions of NOS isoforms in HCMC to the changes in NOS expressions in asthma models suggest that the mast cell is a source of NO in asthmatic airways.

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1. Introduction

Mast cells are pivotal in the pathogenesis of inflammation and nitric oxide (NO) has been suggested to be among the myriads of mast cell derived mediators which levels are increased at sites of inflammation such as asthmatic airways [1]. Transient and low physiological level of NO is synthesized by the constitutive nitric oxide synthases, NOS1 and NOS3 while sustained and high pathological level of NO is synthesized by the inducible NOS2. Despite of studies reporting NO synthesis and the expression of the NO synthesizing enzyme, nitric oxide synthase (NOS) in different models of rat and human mast cells [2,3] there are contradictory reports arguing that mast cells were incapable of NO synthesis and the detections of NOS expression were due to contaminating cells in tissue mast cell preparations or abnormal behaviors of tumor derived mast cell-like cell lines [4,5]. Since there are limited studies on the expression of NOS in primary human mast cells and it is therapeutically important to confirm if these cells are potential cellular sources of NO, we attempted to resolve the current controversy by investigating the NOS expression in pure populations of primary human mast cells cultured from CD34⁺ stem cells isolated from human peripheral blood monocytes.

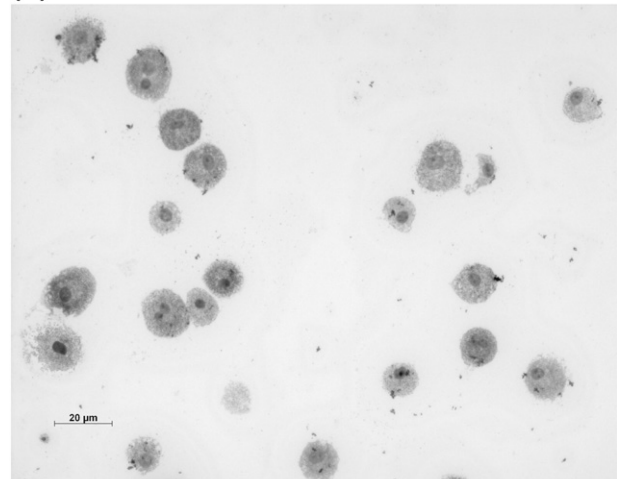
2. Materials and methods

Mature primary human mast cells (HCMC) were derived from progenitors isolated from fresh buffy coat and were morphologically characterised as previously described by ourselves [6]. Briefly, isolated

Table 1 Oligonucleotide primer sequences used for RT-PCR and real-time PCR analysis

Gene	Primer	Sequence (5' → 3')	Position
<i>RT-PCR</i>			
NOS1	Sense	AAGGGCAATGTG-CCTGTCTGT	1486–1506
(U17327)	Antisense	ATTGCCGTTGGC-CTGAAGCA	2288–2268
NOS2	Sense	AGTTTCTGGCAG-CAACGG	3046–3064
(L24553)	Antisense	TTAAGTTCTGTGCC-GGCAG	3578–3561
NOS3	Sense	ACCTGCAAAGCAG-CAAGTCCACG	2303–2325
(AF400594)	Antisense	CCGAACACCAAAG-TCATGGGAGT	3140–3118
<i>Real-time PCR</i>			
NOS1	Sense	GAGCCAGACAAACC-AAAGAAGTAC	3958–3981
(U17327)	Antisense	GCGCTGGATGGCTT-TGAG	4107–4090
NOS2	Sense	CATTGAGATCCCCAA-GCTCTACA	2073–2095
(L24553)	Antisense	TGCCGAGATTTGAG-CCTCAT	2222–2203
NOS3	Sense	GGGCAGCCTCACTC-CTGTT	1359–1377
(AF400594)	Antisense	ACGGCGTTGGCCA-CTT	1508–1493

(A)



(B)

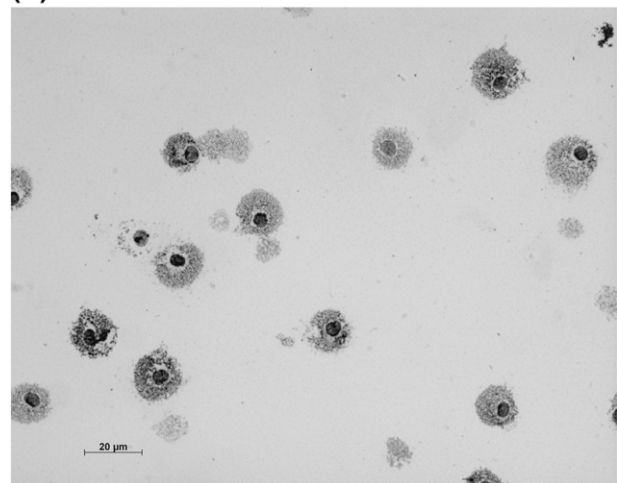


Figure 1 Morphological characterisation of mature primary human cultured mast cells employed in the current study (400×). All cells contain numerous cytoplasmic granules which were stained with (A) May Grunwald and Giemsa stain and (B) positively against monoclonal anti-human tryptase antibody.

progenitors were first cultured with IL-6 and SCF (PeproTech Asia) in serum free methylcellulose (Stem Cell Technologies) for 6 weeks with IL-3 added in the first two weeks only and then in IMDM culture medium [Iscove's modified Dulbecco's medium with 10% foetal bovine serum and 1% penicillin/streptomycin (GIBCO)] supplemented with SCF and IL-6 for 6 weeks. HCMC were characterised with May Grunwald–Giemsa (Merck) staining of cytoplasmic granules as well as immunocytochemical staining of human mast cell tryptase employing monoclonal anti-human tryptase antibody (Chemicon) and the alkaline phosphatase anti-alkaline phosphatase (APAAP) kit from Dako as instructed by the manufacturer. 0.5×10^6 of HCMC were incubated with a cytokines mixture containing 100 ng/ml IFN- γ , 100 ng/ml TNF- α and 10 ng/ml IL-1 β (PeproTech Asia) or with 1 μ g/ml human myeloma IgE (Calbiochem) in IMDM culture medium overnight (16–18 h) at 37 °C. For activation via the IgE receptor (Fc ϵ RI), IgE sensitised HCMC were incubated with anti-IgE (Sigma) at 37 °C for 2 or 6 h. For positive control, the human mast cell line HMC-1, established from a patient with mast cell leukaemia, were obtained from Dr J.H. Butterfield, Mayo Clinic, Rochester, MN, USA [7] and were maintained in Iscove's modified Dulbecco's medium with 10% foetal bovine serum, 2 mM glutamine (GIBCO) and 1% penicillin/streptomycin.

For PCR analysis, RNA was extracted with the Qiagen RNeasy mini kit (Qiagen) and was quantified by measuring the ratio of $A_{260\text{ nm}}/A_{280\text{ nm}}$. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed in a PTC-200 Peltier thermal cycler (MJ Research) using primer pairs (Table 1) and PCR conditions previously described by Gilchrist et al. [3] with the exceptions that optimized annealing temperatures were 60 °C, 57 °C, 53 °C and 55 °C for NOS1, NOS2, NOS3 and GAPDH respectively. Changes in gene expressions of the NOS isoforms were further analyzed with real-time RT-PCR using the Power SYBR Green PCR master mix and ABI Fast Real-Time PCR 7500 system (Applied Biosystems). 0.5 µg of the total RNA was reverse transcribed into cDNA for 30 min at 50 °C by SuperScript(tm) III First-Strand Synthesis SuperMix (Invitrogen). Primer pairs for each gene as listed in Table 1 were designed using the Primer Express 3.0 software program (Applied Biosystems). Thermal-cycling conditions included initial-sample incubation at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The cycle-threshold values (Ct) indicate that the quantity of the target gene in each sample was determined and the comparative Ct ($\Delta\Delta\text{Ct}$) method with GAPDH gene

as the normalizer was used to calculate the relative expressions of NOS genes with reference to control untreated HCMC [8].

Expression of NOS proteins was detected with immunofluorescence staining as described by Wong et al. [9]. Cytoentrifuge smear of same mast cell samples for RT-PCR analysis was incubated with mouse anti-human NOS antibodies (BD Biosciences) and probed with Cy2-conjugated goat anti-mouse IgG secondary antibodies (Jackson ImmunoResearch).

3. Results

HCMC employed in the current study demonstrated morphological and phenotypic properties we previously reported [6,10]. In general, 99% to 100% of the HCMC harvested after 12 weeks culture expressed cytoplasmic granules that stained positively with May Grunwald-Giemsa and immunochemically for human mast cell tryptase (Fig. 1). Untreated, IgE sensitised and cytokine treated HCMC released similar basal levels of histamine which were respectively $7.6 \pm 1.3\%$, $8.2 \pm 1.4\%$ and $7.2 \pm 1.8\%$ of total cellular histamine (mean $\pm$ SEM, $n=3$). Upon activation with anti-IgE (1/1000 dilution), only IgE sensitised HCMC were able to release further $35.7 \pm 3.6\%$ of total cellular histamine (mean $\pm$ SEM, $n=3$).

Similar to the observations reported by Gilchrist et al. [3], untreated HMC-1 expressed NOS1 and NOS3 but not NOS2 in our preliminary conventional RT-PCR analyses. However, expressions of NOS mRNAs were not detected in untreated HCMC (Fig. 2). Real-time RT-PCR detected only marginal levels of NOS2 and NOS3 mRNAs after 33 cycles in untreated HCMC and NOS1 mRNA expression was not detected even after 40 cycles. The expression levels of both NOS2 and NOS3 mRNAs were significantly increased to 2.2 ± 0.6 and 2.8 ± 0.5 folds respectively following overnight incubation of HCMC with IgE (Fig. 2). Following activation with anti-IgE antibody, the expression of NOS2 mRNA continued to increase to 2.8 ± 0.8 and 5.4 ± 1.1 folds after 2 h and 6 h respectively while the expression of NOS3 mRNA was reduced to 2.3 ± 0.6 fold and then to insignificant level respectively. The expression of NOS2 mRNA was also increased to 3.0 ± 0.5 fold after overnight incubation of HCMC with the proinflammatory cytokines mixture (100 ng/ml IFN- γ , 100 ng/ml TNF- α and 10 ng/ml IL-1 β) but not the expression of NOS1 and NOS3. As illustrated in Fig. 3, similar patterns of

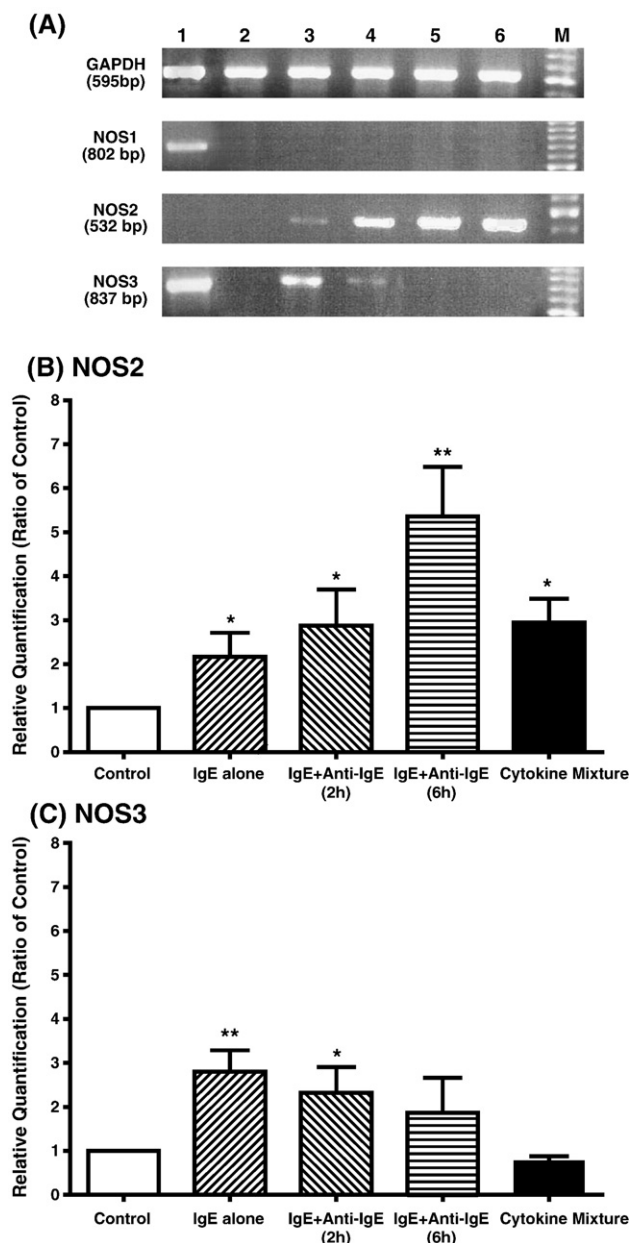


Figure 2 Expression of nitric oxide synthase (NOS) mRNAs in primary human cultured mast cell (HCMC). (A) RT-PCR analysis of NOS1 (802 bp), NOS2 (532 bp) and NOS3 (837 bp) transcripts were performed with primer pairs listed in Table 1. HMC-1 cells were included as the positive control and the PCR product for the GAPDH gene was taken as the reference cellular transcript. Lane numbers are corresponding to: 1. HMC-1 cells, 2. HCMC incubated with culture medium only, 3. HCMC sensitised by incubation with human myeloma IgE overnight (1 µg/ml), 4. sensitised HCMC activated with 1/1000 dilution of anti-IgE for 2 h, 5. sensitised HCMC activated with 1/1000 dilution of anti-IgE for 6 h, 6. HCMC incubated overnight with the proinflammatory cytokine mixture (100 ng/ml IFN- γ , 100 ng/ml TNF- α and 10 ng/ml IL-1 β). (B) and (C) Quantitative gene expressions of respectively NOS2 and NOS3 were performed on the ABI Fast Real-Time PCR 7500 system using primer pairs as listed in Table 1. The comparative Ct method ($\Delta\Delta\text{Ct}$) with GAPDH gene as the normalizer was used to calculate the relative quantification values which were expressed as a ratio of gene expression in the untreated control HCMC. Statistical significance analysis of the relative expression of NOS genes was performed by subjecting the ΔCt values for treatment and control samples to simple *t*-test as recommended in Ref. [7]. Each value represents mean $\pm$ SEM for 5 experiments. Significant differences between the control and treated HCMC are indicated by asterisks: * $P < 0.05$ and ** $P < 0.01$.

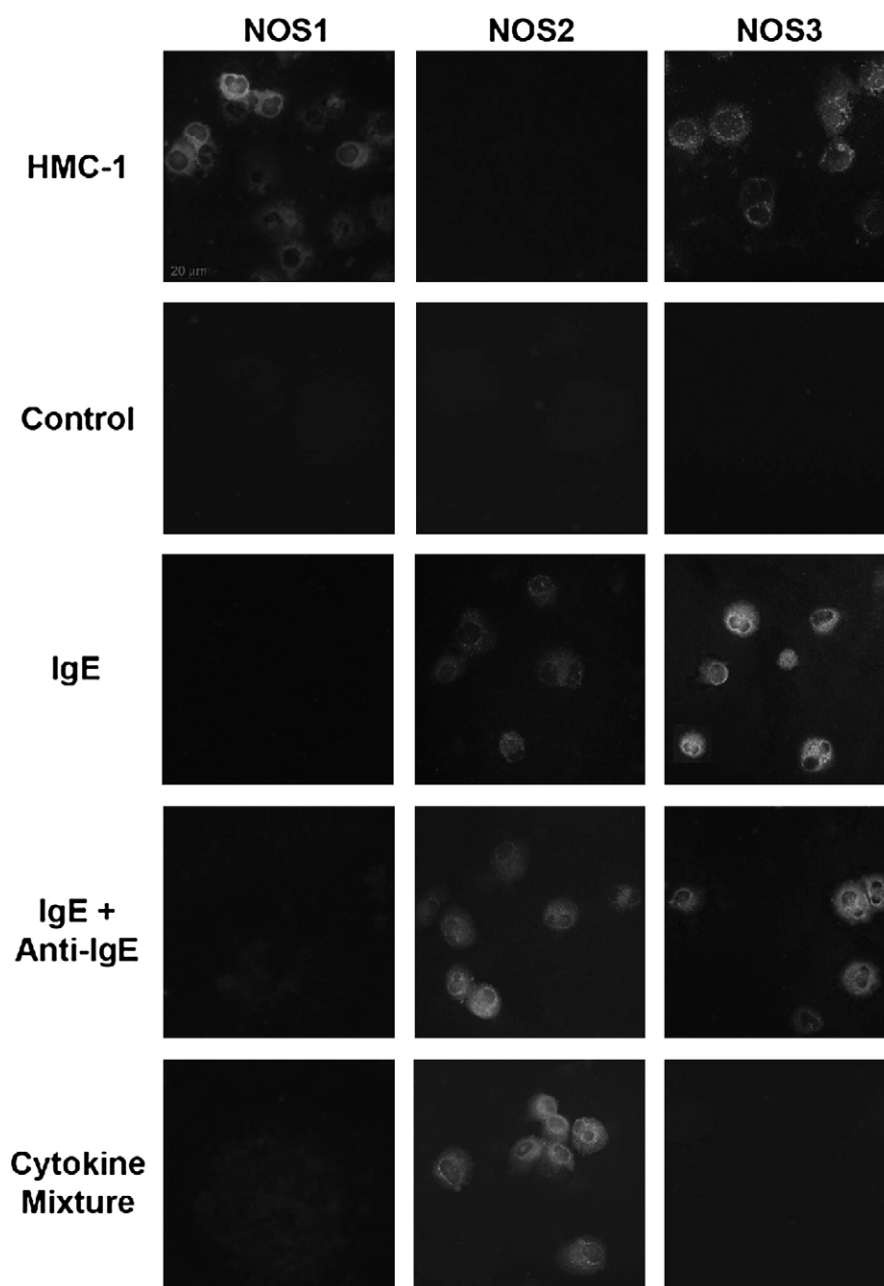


Figure 3 Immunofluorescence images of NOS expression in the positive control HMC-1 cells, unstimulated HMC (control), HMC incubated overnight with 1 μ g/ml human myeloma IgE (IgE), IgE sensitised HMC 6 h after activation with 1/1000 dilution of anti-IgE (IgE + anti-IgE) and HMC incubated overnight with a mixture of 100 ng/ml IFN- γ , 100 ng/ml TNF- α and 10 ng/ml IL-1 β (cytokines mixture). NOS antibodies were detected with Cy2-conjugated secondary antibodies and shown in green fluorescence. Fluorescent images were captured with a Zeiss Ax fluorescent microscope (Carl Zeiss) at a magnification of 400 $\times$. No expression of NOS1 protein was detected in HMC while highest fluorescence intensity for NOS3 and NOS2 was detected in IgE sensitised and anti-IgE activated HMC respectively.

NOS proteins expressions were identified by immunochemical staining and thus further confirmed the RT-PCR results.

4. Discussion

We report here for the first time the induction of both NOS2 and NOS3 expressions upon appropriate stimulations and thus provide indirect evidence that human primary mast cells are potential sources of tissue NO. The absence of NOS1 expression however contradicts with previous reports in

human tissue biopsies [11], partially purified skin mast cells and HMC-1 cells [3]. The different patterns of NOS1 expression may be the consequences of different degree of cell maturity, phenotypic differences among tissue mast cells and influences of endogenous factors which were not tested in our current study.

The increase in NOS2 and NOS3 expressions subsequent to overnight incubation with IgE supported previous reports that binding of monomeric IgE to Fc ϵ RI could initiate signal transductions in mast cells even when degranulation was

absent [12]. The increased NOS expression implicates that human mast cells are capable of increasing the level of NO in the immediate cellular environment following IgE sensitisation. Since local inflammation is not observed when tissue mast cells are sensitised, the level of NO produced at this stage would be relatively low but adequate to maintain local tissue homeostasis. The failure of inducing NOS3 expression by the proinflammatory cytokines and the reduction of NOS3 expression subsequent to mast cell degranulation suggest that NOS3 may be the major NOS regulating NO production of sensitised mast cells. In fact, NOS3 was shown to be associated with the regulation of airway tone in antigen sensitised animals [13] and the limitation of bradykinin induced bronchoconstriction in patients with mild asthma [14]. Furthermore, it was demonstrated that constitutive NOS derived NO level in exhaled air was transiently increased within the first 30 min of antigen challenge and played an important inhibitory modulating function during the early phase of asthma reaction [15]. With the expression of NOS3 up-regulated during sensitisation and the increase in intracellular calcium following antigen challenge, our results suggest that airway mast cells contribute to this early rise in bronchoprotective level of NO.

In animal models of asthma and patients with mild asthma, NOS3 expression in the airway is reduced 30 min after antigen provocation and subsequently the reactivity of airways increases with a concomitant increase in the expression of NOS2 [15,16]. The development of late phase asthma and the severity of chronic asthma have been shown to be related to increased levels of exhaled NO synthesized by NOS2 [15,16]. The upregulation of NOS2 in HCMC following activation by anti-IgE/IgE reaction and cytokines and the continual increase following anti-IgE/IgE reaction suggest that mast cells would be the first local inflammatory effector cells to produce high level of NOS2 derived NO that initiates and sustains local tissue inflammation. Similar to the pattern of NOS isoforms changes in the development of asthma, our observation of the NOS3 decrease accompanying the NOS2 increase subsequent to IgE/anti-IgE reaction suggests that regulatory roles of NOS3 responsible for low physiological level of NO released by sensitised mast cells are superseded by proinflammatory functions of NOS2 responsible for high pathological level of NO synthesized by activated mast cells. The reduction in the expression of NOS3 is possibly a prerequisite for NOS2 induction since physiological level of constitutive NOS derived NO has been reported to inhibit NOS2 transcription by suppressing NF- κ B activation [17]. The reduction in the expression of NOS2 and NOS3 may also be a consequence of the autocrine effects of NO which have been suggested to produce a negative feedback control on mast cell activation [18].

In summary, although direct measurement of NO levels was not performed in the current study, we have produced indirect evidence supporting that human mast cells are one of the cellular sources of NO in inflammatory conditions such as asthma. The differential expressions of NOS3 in IgE sensitised mast cells but not in cytokine activated mast cells suggest that different signaling pathways are activated by the two stimuli. It will hence be useful to delineate these pathways in human cultured mast cells for developing anti-asthmatic agents that specifically block only the expression of NOS2 but not NOS3, i.e. suppressing the generation of high proinflammatory level of NO but retaining the synthesis of low bronchoprotective level of NO.

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