Homocysteine enhances superoxide anion release and NADPH oxidase assembly by human neutrophils. Effects on MAPK activation and neutrophil migration

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Abstract

Hyperhomocysteinaemia has recently been recognized as a risk factor of cardiovascular disease. However, the action mechanisms of homocysteine (Hcy) are not well understood. Given that Hcy may be involved in the recruitment of monocytes and neutrophils to the vascular wall, we have investigated the role of Hcy in essential functions of human neutrophils. We show that Hcy increased superoxide anion (O2•−) release by neutrophils to the extracellular medium, and that this effect was inhibited by superoxide dismutase and diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity. The enzyme from rat peritoneal macrophages displayed a similar response. These effects were accompanied by a time-dependent increased translocation of p47phox and p67phox subunits of NADPH oxidase to the plasma membrane.

We also show that Hcy increased intracellular H2O2 production by neutrophils, that Hcy enhanced the activation and phosphorylation of mitogen-activated protein kinases (MAPKs), specifically p38-MAPK and ERK1/2, and that the migration of neutrophils was increased by Hcy. Present results are the first evidence that Hcy enhances the oxidative stress of neutrophils, and underscore the potential role of phagocytic cells in vascular wall injury through O2•− release in hyperhomocysteinaemia conditions.

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1. Introduction

Leukocyte-endothelial cell interactions are crucial events in inflammatory atherosclerosis. In early stages of atherosclerosis, monocytes and neutrophils infiltrate the intima and generate reactive oxygen species (ROS) which provoke oxidative damage to endothelial cells [1–3]. However, the precise mechanisms of neutrophil activation remain unknown. Homocysteine (Hcy) is a thiol-containing amino acid derived from the metabolism of methionine. Hyperhomocysteinaemia (defined by values of plasma Hcy >15 μM) has been recognized as an independent risk factor for atherosclerosis, coronary, cerebral and peripheral arterial disease, and venous thromboembolism [4,5]. However, other authors have pointed out that factors other than Hcy could be causative of atherosclerosis, and have argued that the elevation of blood Hcy levels could be a consequence rather than a cause of these diseases [6–8].

In any event, the mechanisms underlying the association between hyperhomocysteinaemia and increased vascular risk are not well understood. Most hypotheses involve injury to the vascular endothelium or some alteration in endothelial function [9,10], suggesting that Hcy contributes to enhanced vascular inflammation. The presence of high levels of circulating Hcy may affect not only endothelial cells, but also immune cells. Recently, it has been reported that
Hcy increases the interactions of neutrophils [11], monocytes and T-cells with endothelial cells [12], and also that Hcy induces the expression of monocyte tissue factor [13]. In this context, it is known that neutrophils induce the release of endothelial monocyte-chemoattractant protein-1 [14,15]. It has also been suggested that Hcy acts indirectly through its oxidation and the concomitant production of ROS [16]. On the other hand, the synthesis of $O_2^{•−}$ and ROS has been demonstrated to be a key functional response by neutrophils. Phagocytic cells exhibit a potent NAPDH oxidase activity responsible for the synthesis of $O_2^{•−}$ [17]. Until now, an effect of Hcy on $O_2^{•−}$ and ROS production by phagocytic cells has not been reported. In this paper we show that Hcy increases $O_2^{•−}$ release by phagocytic cells, and that this effect is accompanied by the translocation of $p47^{phox}$ and $p67^{phox}$ subunits of NADPH oxidase to the plasma membrane. Furthermore, neutrophils enhanced $H_2O_2$ production in response to Hcy. Given that exogenous oxidants are known to promote phosphorylation, and hence activation of MAPKs in human neutrophils [18–20], we set out to analyze whether Hcy-dependent $O_2^{•−}$ production is linked to MAPK activation, specifically of $p38$-MAPPK and ERK1/2, in these cells. The results obtained indicated that Hcy promotes phosphorylation of both MAPKs in human neutrophils, together with a concomitant decrease in the levels of MAPK phosphatase-1 (MKP-1), a protein which specifically dephosphorylates both $p38$-MAPK and ERK1/2 [21]. We also report that Hcy stimulated the migration of human neutrophils. Present observations constitute the first evidence of a direct effect of Hcy, at pathophysiologically relevant concentrations, on the activation of oxidative stress of phagocytic cells, and add new insight into the potential participation of these phagocytic cells in Hcy effects on the vascular wall.

2. Methods

2.1. Materials

$\alpha$-homocysteine, diphenyleneiodonium (DPI), diethylidithiocarbamate (DDTC), superoxide dismutase (SOD), ILMP, and other biochemical reagents were obtained from Sigma-Aldrich (Madrid, Spain). Rabbit polyclonal anti-phospho-specific p38-MAPK (Thr180/Tyr182) (anti-p-p38-MAPK), mouse monoclonal anti-phospho-specific ERK1/2 (Thr202/Tyr204) (anti-p-ERK1/2) and antibodies against their unphosphorylated counterparts, as well as rabbit polyclonal antibody against MKP-1 were kindly donated by T.L. Leto (NIH, Bethesda, MD). Costar Transwell tissue culture inserts were from Corning Costar Corp (Cambridge, MA, USA).

2.2. Isolation and culture of human neutrophils and peritoneal rat macrophages

Neutrophils were obtained from fresh heparinized blood of healthy human donors, after informed consent, by dextran sedimentation followed by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden)-gradient centrifugation and hypotonic lysis of residual erythrocytes as described previously [22]. Assays with adherent neutrophils were conducted in plates coated with saturating concentrations of fibrinogen (2 mg/ml). Cells were incubated for 3 h at 37 °C, washed three times to remove nonadherent cells, and finally resuspended in fresh medium. Peritoneal macrophages were isolated from rat by peritoneal lavage [23]. Since Hcy in aqueous solution is readily lost by oxidation [11], we added Hcy every hour in those experiments lasting more than 1 h.

2.3. Assay of $O_2^{•−}$ production

The levels of $O_2^{•−}$ were analyzed by the lucigenin-based chemiluminescence method. Cells were suspended in Hepes-buffered Krebs–Ringer solution (KR–Hepes) at $10^6$ cells/ml and preincubated at 37 °C for 5 min. Then, $15 \mu M$ lucigenin (Sigma-Aldrich) was added and the assay was carried out as indicated [24]. Because lucigenin permeates freely through the cell membranes, its luminescence was an indication of intra- plus extracellular ROS. Hcy was added to the cell suspension, and chemiluminescence emission was recorded at different times using a BioOrbit 1250 luminometer.

2.4. Mobilization of $p47^{phox}$ and $p67^{phox}$ subunits to plasma membrane

Translocation to membrane of $p47^{phox}$ and $p67^{phox}$ NADPH oxidase subunits was analyzed as previously reported [24], with minor modifications, and the membrane levels of the $p22^{phox}$ subunit were monitored as a control, following standard protocols by Mankelow and Henderson [25]. Briefly, neutrophils were resuspended in relaxation buffer, consisting of 10 mM Tris–HCl (pH 7.3), 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl$_2$, 1.25 mM EGTA, 1 mM ATP, 2 mM sodium orthovanadate, 100 $\mu$M phenylarsine oxide, 3 mM disopropyl fluorophosphate, 10 $\mu$g/ml leupeptin, and 10 $\mu$g/ml aprotinin, and disrupted by sonication on ice. After ultracentrifugation at 100,000 $\times$ g for 30 min at 4 °C, the membrane pellet obtained was resuspended in solubilization buffer, consisting of 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P40, and 0.1% SDS, and then recentrifuged at 20,000 $\times$ g for 40 min at 4 °C. The supernatant obtained, containing solubilized membranes, was boiled in Laemmli loading buffer. Proteins $p67^{phox}$, $p47^{phox}$ and $p22^{phox}$ were detected by Western blotting analysis using specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies, followed by blot development using an enhanced chemiluminescence assay [26].
2.5 Analysis of $O_2^•−$ levels

2.7-Dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich) was used as an indicator of the amounts of intracellular $H_2O_2$ [27]. Briefly, $5 \times 10^6$ neutrophils were suspended in 1 ml of KR-Hepes and incubated at 37°C for 1 h in the dark in the presence of 2.5 μM DCFDA, 2.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aproamin, 2 mM sodium orthovanadate, and 100 μM phenylarsine oxide, and disrupted by sonication on ice. Insoluble material was removed by centrifugation at 12,000 g. The cells were then rinsed twice with KR-Hepes plus 2.5 mM phenylmethylsulfonyl fluoride and 2.5 mM 3-amino-1,2,4-triazole prior to the addition of Hcy, and the fluorescence intensity was measured for 30 min in a Wallac 1420 VICTOR2 spectrophotometer, using excitation and emission wavelengths of 485 and 535 nm, respectively.

2.6 Analysis of p38-MAPK and ERK-1/2 phosphorylation, and MKP-1 protein levels

The cells were resuspended in a lysis solution containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 50 mM DTT, 50 mM NaF, 1% Nonidet-P40, 1% Triton X-100, 3 mM diisopropy lfluorophosphate, 10 μg/ml leupeptin, 10 μg/ml aproamin, 2 mM sodium orthovanadate, and 100 μM phenylarsine oxide, and disrupted by sonication on ice. Insoluble material was removed by centrifugation at 12,000×g at 4°C, and the supernatant obtained was boiled in Laemmli loading buffer. MKP-1 protein and phosphorylated isoforms of p38-MAPK and ERK1/2 were detected by Western blotting (see above) using specific antibodies. To verify even protein loading, the blots were sub sequently stripped and reprobed with antibodies against β-actin.

2.7 Chemotaxis assay

Migration of neutrophils was performed in Transwell chambers (6.5 μm diameter, 5 μm pore size, type 3421). Chemotactants were deposited in the lower compartments in a final volume of 0.6 ml of RPMI 1640, and prewarmed at 37°C. Then 0.1 ml of medium containing 10^6 neutrophils was deposited into the detachable inserts, which were placed over the chemotactant solutions. Loaded chambers were incubated for 2 h at 37°C in a humidified atmosphere of 5% CO2 in air. In separate wells, neutrophils were added to the lower compartment and used as control of 100% migration. At the end of incubation, the cells that had migrated into the bottom chambers were collected and centrifuged. After staining with FITC-conjugated monoclonal antibodies to CD16 (Immunotech, Marseille, France), the cells were fixed with 1% paraformaldehyde and finally counted on a flow cytometer. The results are presented as the mean ± S.E.M. of three separate experiments, and are expressed as the percentage of total neutrophils initially added to the chamber.

2.8 Other analytical procedures

Assays of intracellular reduced glutathione (GSH) levels [28] and lysozyme release [29] were performed as previously described.

3. Results

3.1 Homocysteine induced $O_2^•−$ release and promoted the translocation to the plasma membrane of p47phox and p67phox subunits of NADPH oxidase

Since Hcy-dependent generation of ROS has been widely postulated as a mechanism for Hcy potentiating actions on endothelial cells [11,30], we used as a working hypothesis that NADPH oxidase from phagocytic cells could play a role in vascular wall injury during the hyperhomocysteinemic state. To test this possibility, human neutrophils were incubated in the presence of Hcy doses ranging between 10 and 500 μM. Fig. 1A illustrates that Hcy induced a dose- and time-dependent increase of $O_2^•−$ production, which was still increasing at the highest concentration tested (500 μM). At low (10 μM) Hcy doses appreciable amounts of O2•− were produced, and concentrations above 10 μM increased the O2•− release by 5–10 fold as compared to that measured in unstimulated neutrophils. The effect of phospholipid 12-myristate 13-acetate (PMA), a well-known activator of NADPH oxidase, was also analyzed to ascertain the functional responsiveness of neutrophils. Measured amounts of O2•− production were 6.1 ± 2.3 luminescence relative units (l.r.u.) in control, unstimulated cells, 122 ± 3 luminescence units (l.r.u.) in 100 nM PMA-treated cells (data are expressed as the mean ± S.E.M. of three experiments). Hence, 100 nM PMA elicited a higher $O_2^•−$ production by neutrophils than did 500 μM Hcy. Besides, when before Hcy treatment neutrophils were preincubated with DPI, an inhibitor of NADPH oxidase activity [31], a dose-dependent inhibition of $O_2^•−$ production was observed (Fig. 1B), thus indicating the occurrence of Hcy-elicited NADPH oxidase activation. Subsequently, we investigated whether Hcy-dependent O2•− production could be altered when human neutrophils were preincubated with SOD, a scavenger of O2•−. We observed that SOD addition to neutrophils before Hcy treatment cancelled the Hcy-dependent O2•− production (Fig. 1C). It should be noted that the use of the cytochrome c assay to analyze extra cellular O2•− released by Hcy was not feasible, because Hcy was itself able to directly reduce cytochrome c. In control experiments, it was verified that Hcy by itself did not elicit lucigenin–chemiluminescence production (data not shown).

In summary, Hcy induced in human neutrophils O2•− production in an associated manner to the activation of phagocytic NADPH oxidase.

The translocation of p47phox and p67phox subunits of NADPH oxidase from the cytosol to the plasma mem-
Homocysteine increases O$_2^•−$ release by human neutrophils. Cells were incubated at 37 °C under the following conditions: (A) with medium alone or supplemented with increasing doses of Hcy; (B) preincubated with solvent, dimethylsulfoxide (DMSO), (open symbols) or with increasing doses of DPI (filled symbols) for 5 min, and then treated with 500 μM Hcy. Lucigenin (15 μM) was added prior to Hcy, and released O$_2^•−$ was measured for 10 min after Hcy addition on the basis of lucigenin chemiluminescence, as indicated in Materials and Methods. The mean of l.r.u. is luminescence relative units. Plotted values are the mean ± S.E.M. from three separate experiments in which each measurement was performed in triplicate.

brane is an essential step for the activation of NADPH oxidase [32]. In order to further ascertain the involvement of this enzyme in Hcy-dependent O$_2^•−$ production, membrane levels of p47$^{phox}$ and p67$^{phox}$ were evaluated in Hcy-treated neutrophils. Fig. 2A illustrates that Hcy enhanced translocation of both NADPH oxidase subunits to the plasma membrane. This effect was apparent at 5 min of incubation with Hcy for both p47$^{phox}$ and p67$^{phox}$. However, after 30 min of treatment although the Hcy effect was still evident for p67$^{phox}$, p47$^{phox}$ membrane levels had decreased. The membrane levels of p22$^{phox}$, in keeping with it being an integral membrane protein, were...
similar in proportions from both untreated and Hcy-treated cells. Most studies of $\text{O}_2^{•−}$ production by neutrophils have been carried out on cells in suspension. However, in a more physiological scenario neutrophils are known to adhere to endothelial cells or basement membrane components at sites of inflammation[33]. To analyze whether Hcy could also affect neutrophil adherence, Hcy-induced translocation of p47$^{\text{phox}}$ and p67$^{\text{phox}}$ was assessed in human neutrophils attached on fibrinogen-coated wells. Positive results were also obtained in these cells for both subunits of NADPH oxidase (Fig. 2B).

To assess whether Hcy-dependent NADPH oxidase stimulation was restricted to human neutrophils, experiments using rat peritoneal macrophages were also performed in which these cells were incubated with Hcy and luminescence arising from $\text{O}_2^{•−}$ production was recorded. Hcy induced a dose- and time-dependent increase of $\text{O}_2^{•−}$ release, which was still increasing at the highest dose studied, 500 $\mu$M (Fig. 3A). We found that only at Hcy doses higher than 10 $\mu$M was there a significant increase in the production of $\text{O}_2^{•−}$ as compared with non-stimulated cells, albeit at 10 $\mu$M Hcy such increase was already appreciable. Also in parallel with human neutrophils, when rat macrophages were preincubated with DPI, a clear inhibition of Hcy-dependent $\text{O}_2^{•−}$ production was obtained, till becoming abolished at 1 $\mu$M DPI (Fig. 3B).

3.2. Homocysteine induced $\text{H}_2\text{O}_2$ production in human neutrophils

Having shown the increase in $\text{O}_2^{•−}$ levels following Hcy treatment, we next addressed the issue of possible alterations of other parameters involved in oxidative stress, such as $\text{H}_2\text{O}_2$ production. In this light, increased $\text{H}_2\text{O}_2$ levels in endothelial cells in response to Hcy have been previously reported [9,30]. Fig. 4 illustrates that Hcy added to human neutrophils at doses ranging between 10 and 250 $\mu$M also enhanced intracellular $\text{H}_2\text{O}_2$ production in a dose- and time-dependent manner. It is also noteworthy that upon manipulation and incubation of cells without additions, production of measurable amounts of intracellular $\text{H}_2\text{O}_2$ took place (Fig. 4), which was in contrast with the negligible $\text{O}_2^{•−}$ amounts released by neutrophils when NADPH oxidase was not activated (Fig. 1A and C). Although most of the detected $\text{H}_2\text{O}_2$ production should arise from the dismutation of $\text{O}_2^{•−}$ other intracellular processes also leading to $\text{H}_2\text{O}_2$ synthesis could become unspcifically activated. Amongst the systems used by the cell to protect itself against oxidative damage is biasing the oxidized/reduced glutathione (GSSG/GSH) balance towards GSH. It has been shown that only at levels greater than those observed in hyperhomocysteinemic patients (e.g. 5 mM, but not 50 $\mu$M) does Hcy have a stimulating effect on intracellular GSH [34]. We thus set about testing whether Hcy (100 and 250 $\mu$M) could have
some effect on this antioxidant system in neutrophils. We found that Hcy did not exert any effect on GSH intracellular levels or alter the release of lysozyme (data not shown), a protease present in secretory vesicles of neutrophils which is secreted in inflammatory conditions [35].

3.3. Homocysteine activated MAPK in human neutrophils

It has recently been reported that neutrophils use the p38-MAPK and ERK1/2 cascade to link a variety of agonist-promoted activities to an array of functional responses [18,20]. In this context, we investigated the possible relationship between Hcy-dependent O$_2^•−$ production and activation of p38-MAPK and/or ERK1/2, and explored the potential participation of these kinases in the transduction of the Hcy signal in human neutrophils. Toward this end, we analyzed the phosphorylation status of these two MAPKs by means of Western blotting using phospho-specific antibodies. As shown in Fig. 5A, phosphorylation levels of p38-MAPK were maximal after 30 min of treatment with 100 μM Hcy, and remained high for at least 60 min. Hcy was also able to promote phosphorylation of ERK1/2, which also reached maximal levels after 30 min of Hcy treatment. The activity of both MAPKs is regulated via reversible phosphorylation of tyrosine and threonine residues by specific protein phosphatases, of which one of them, MKP-1, is ubiquitously expressed and is known to dephosphorylate both ERK1/2 and p38-MAPK [21]. We thus analyzed whether Hcy affected the levels of MKP-1 protein. Fig. 5A illustrates that after 15–30 min of treatment with Hcy, the intracellular amount of MKP-1 protein clearly decreased with respect to that found in untreated cells. Longer times of treatment (60 min) resulted in slow restoration of basal levels of this protein. To verify the specificity of this response, we used two specific compounds: PD098059, which inhibits MEK1/2, the upstream activator of ERK1/2 [36], and SB203580, which inhibits the kinase activity of p38-MAPK [37]. Fig. 5B illustrates that the Hcy-induced phosphorylation of ERK1/2 could be partially reduced by PD098059. The addition of SB203580 had the same effect on p38-MAPK phosphorylation (data not shown). The simultaneous presence in these assays of Hcy and DPI, an inhibitor of NADPH oxidase and other flavins [31], or of DDTC, a radical scavenger, had no effect on the Hcy-promoted phosphorylation of either p38-MAPK or ERK1/2 (Fig. 5C). These facts suggest that Hcy-induced MAPK activation can occur in a manner independent of ROS production.

3.4. Migration of human neutrophils in response to homocysteine

Data have been presented supporting a positive relationship between atherosclerosis and neutrophil activation...
and migration [14,15]. In this light, neutrophil migration experiments were performed in which cells were incubated for 2 h with Hcy or fMLP, a known chemotactic factor for neutrophils [38], and the cells that had migrated through membranes during that period were labelled with FITC-conjugated anti-CD16 antibodies and counted by flow cytometry. Fig. 6 shows that the level of neutrophil migration induced by Hcy was almost three-fold that found in untreated neutrophils, and similar to that obtained with fMLP.

4. Discussion

Over the past years, homocysteine has emerged as a potential risk factor for premature cardiovascular disease, promoting many processes that play a role in vascular and endothelial cell damage [39]. Thus, it has been reported that a progressive increase in the plasma Hcy concentration raises the risk of coronary artery disease [40]. In addition, when laboratory animals were infused with Hcy, their arteries showed a thicker intima, proliferation of their smooth muscle cells, increased decalcification of the luminal surface and high levels of foam cells [41]. However, it has also been suggested that factors other than Hcy can cause atherosclerosis, and that elevation of Hcy levels is just a consequence and not a cause of these diseases [6–8]. Although normal levels of Hcy in fasting conditions are of 5–15 μM, in cases of hyperhomocysteinaemia they may reach values of 16–30 μM (moderate), 31–100 μM (medium) or greater than 100 μM (severe), values as high as 500 μmol/l being found in patients with homocystinuria [7]. The complexity of relationship between Hcy levels and cardiovascular diseases may be envisaged by the observation of a multiplicative increase in the risk of vascular disease in the presence of traditional risk factors and hyperhomocysteinaemia [42]. Thus, a significant correlation has been shown between the concentration of plasma Hcy and hypertension [43], and also an Hcy-promoted increase in the formation of highly atherogenic oxysterols, lipid peroxidation and oxidation of low-density lipoprotein in vitro [44].

However, the mechanisms underlying endothelial injury promoted either by Hcy alone or in combination with other risk factors are not well understood. In endothelial cells, in vitro studies provide evidence that supraphysiological Hcy levels have a direct toxic effect, probably through free-radical generation through oxidation of the Hcy sulphhydril group [45]. The present study addresses the hypothesis that deleterious effects of Hcy on the vascular wall could be mediated by the oxidative stress promoted
that \( \text{H}_2\text{O}_2 \) generated by neutrophils and/or endothelial cells. We have found that Hcy significantly increased \( \cdot\text{O}_2^- \) on the activation of NADPH oxidase in human neutrophils. Hcy-induced endothelial-cell injury was largely secondary to inflammatory events occurring in the vascular endothelium, in addition to neutrophils. However, the specific mechanism of NADPH oxidase activation in neutrophils by Hcy needs still to be clarified. Present data also indicate that Hcy stimulates the respiratory burst in other phagocytic cells, such as macrophages involved in the inflammatory events occurring in the vascular endothelium, in addition to neutrophils.

Further, the increase in \( \cdot\text{O}_2^- \) release induced by Hcy was accompanied by an increase of \( \text{H}_2\text{O}_2 \) production. Different sources and pathways for \( \text{H}_2\text{O}_2 \) generation exist in eukaryotic cells. In this light, mitochondria, microsomes, peroxisomes and cytosolic enzymes have been recognized as effective generators of ROS, which after dismutation yield \( \text{H}_2\text{O}_2 \). For instance, there is evidence that \( \text{H}_2\text{O}_2 \) in mitochondria is derived from the dismutation of \( \text{O}_2^\ast^- \) generated by minor side reactions from the sequential reduction of \( \text{O}_2^\ast^- \) via respiratory carriers [47]. Recently, it has been suggested that \( \text{H}_2\text{O}_2 \) generated by neutrophils and/or endothelial cells during interaction between these two cell types is involved in Hcy-dependent vascular endothelium damage [11]. However, this is an indirect assumption based on the fact that the addition of catalase as an \( \text{H}_2\text{O}_2 \) scavenger is able to block the Hcy-mediated neutrophil-endothelium interaction. Earlier works have demonstrated that Hcy undergoes in vitro oxidation to homocystine and that \( \text{H}_2\text{O}_2 \) is generated in this process [9,30]. In these reports it has been concluded that Hcy-induced endothelial-cell injury was largely secondary to \( \text{H}_2\text{O}_2 \) generation. Our present data indicate that the potential participation of neutrophils in vascular wall injury is mediated mainly by \( \cdot\text{O}_2^- \) although the participation of \( \text{H}_2\text{O}_2 \) could take place as previously shown [9,30].

Another goal of this study was the possible role of Hcy-induced ROS generation in the activation of kinases of the MAPK family in human neutrophils. It has been previously demonstrated that Hcy stimulates MAPK activation in smooth muscle cells [48] and, furthermore, to cause activation of c-Jun NH(2)-terminal kinase in vascular endothelial cells [49]. Our findings in neutrophils are in agreement with these results, and underscore the potential role that neutrophils may play as target cells in the hyperhomocysteinemic state. The relationship between ROS and agnostin-dependent activation of the different forms of MAPK in neutrophils has been previously dealt with [18]. Present data illustrate that prooxidative conditions promoted by Hcy are partially coupled to the activation of p38-MAPK and ERK1/2, although the exact hierarchical order of Hcy-dependent activation of NADPH oxidase and MAPKs needs yet to be clarified. At the same time, adhesion and chemotaxis of phagocytic cells is a key initial step to inflammation in the vascular wall [2,50]. Our work also shows that Hcy induces chemotaxis in isolated neutrophils. The increase in the number of polymorphonuclear cells migrating in response to Hcy was similar to that elicited by fMLP, a well-established chemotactic inductor of neutrophils [38]. Recently, proof has been presented that Hcy selectively induces surface changes in neutrophils leading to their increased adhesion to human endothelial cells [11].

Several questions arise from the present observations, notably on the specificity of the effect of Hcy on phagocytic cells and its physiological relevance. It should be noted that dt-Hcy was used in our experiments, and have found a dose of \( 100\mu\text{M} \) of Hcy to induce a marked increase in \( \cdot\text{O}_2^- \) release. It may be speculated that, in the case that only the t-stereoisomer was active, the effective concentration would have been half that reported here. It is worth noting that in hyperhomocysteinemic patients, blood Hcy levels range between 16 uM and a few hundred uM, and the doses described in the present study as required to activate NADPH oxidase from neutrophils fall within such range of concentrations. A functional significance of our results may be inferred, in the light that in addition of monocytes, neutrophils have been proposed to play a role in the pathogenesis of atherosclerosis [3,14,50]. The latter is characterized by monocyte infiltration, which can be mediated by neutrophil-induced release of endothelial monocyte-chemoattractant protein-1 [14,15]. Moreover, inappropriate active neutrophils lead to endothelial damage and inflammation of small vessel in systemic vasculitis [51].

Taking present data as a whole, they can be interpreted to reflect the fact that NADPH oxidase activity of phagocytic cells is sensitive to Hcy-induced activation. Therefore, our results on the stimulating effect of Hcy on respiratory burst add new insight into vascular wall damage with elevated plasma levels of Hcy found in coronary artery disease. In this context, they suggest the potentiation of \( \cdot\text{O}_2^- \) release, migration of neutrophils and injury on endothelial cells as a potential mechanism by which Hcy can increase the risk of developing atherosclerosis and other cardiovascular diseases. At present, it is being evaluating whether the reduction of Hcy levels (e.g. by the combination of folic acid and group B vitamins) can provide an easy and low-cost treatment to reduce cardiovascular risk [52]. Present observations could also suggest new investigation protocols on the effects
of antioxidant agents and neutrophil inhibitors in amelio-
rating oxidative stress-dependent atherosclerotic damages in
the vascular wall.

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References

stein NM. Activated polymorphonuclear leukocytes and monocytes
in the peripheral blood of patients with ischemic heart and brain
conditions correspond to the presence of multiple risk factors for

Activation of human neutrophil by cytochrome-activated endothelial

[3] Huang ZS, Jing JS, Wang CH, Yip PK, Wu TH, Lee TK. Corre-
lations between peripheral differential leukocyte counts and careful


[5] Ueland PM, Refsum H, Beresford SA, Vollset SE. The contro-
versy over homocysteine and cardiovascular risk. Am J Clin Nutr


[7] Medina M, Uriarte JL, Amoreo-Sanchez ML. Roles of homocys-
etine in cell metabolism: old and new functions. Eur J Biochem

[8] Cleophas TJ, Hornstra N, Hoopstra R, van der MJ, Homocys-
etine, a risk factor for coronary artery disease or not? A meta-analysis.

endothelial cell injury in vitro: a model for the study of vascular

[10] Hajjar KA. Homocysteine-induced modulation of tissue plasminogen
activator binding to its endothelial cell membrane receptor. J Clin

enhances neutrophil–endothelial interactions in both cultured human

Release of chemoattractants for human monocytes from endothelial


[14] Heyworth PG, Curnutte JT, Nauseef WM, Volpp BD, Pearson DW,
Fahmy MA. Homocysteine: a risk factor for coronary artery disease or not? A meta-

[15] Boyum A. Isolation of mononuclear cells and granulocytes from
human blood. Isolation of mononuclear cells by centrifugation, and
of granulocytes by centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 1968;57:77–89.

activates Rac and Rho in human neutrophils: potential role in activation

inactivation of granulocyte-specific protein phosphatases by
MKP-1 and MKP-2 have unique substrate specificities and reduced activity

[18] Fialkow L, Chan CK, Rotin D, Grinstein S, Downey GP. Activation of
the neutrophil–endothelial protein kinase signaling pathway in neutrophils.

activates Rac and Rho in human neutrophils: potential role in activation

[20] Boyum A. Isolation of mononuclear cells and granulocytes from
human blood. Isolation of mononuclear cells by centrifugation, and
of granulocytes by centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 1968;57:77–89.

[21] Chiara MD, Bedoya F, Sobrino F. Cyclosporin A inhibits phorbol
ester-induced activation of superoxide production in resident mouse

[22] Boyum A. Isolation of mononuclear cells and granulocytes from
human blood. Isolation of mononuclear cells by centrifugation, and
of granulocytes by centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 1968;57:77–89.

J, Pintado E. Activation of phagocytic cell NADPH oxidase by
toxofactor. A potential mechanism to explain its bacterial action.

Oxidative stress is a critical mediator of the angiotensin II signal in
human neutrophils: involvement of mitogen-activated protein kinase,
calcinein, and the transcription factor NF-kB. Blood 2003;102:662–
74.

[25] Cuthbert R, Schweer A, Ames BN. Detection of pimocide levels of
hydroperoxides using a fluorescent dichlorofluorescein assay. Anal

[26] Huisin PJ, Hui R. A fluorometric method for determination of oxy-
26.

[27] Gallin JI, Everett MP, Sellemann HM, Hoffstein S, Cebs K, Moun-
ness N. Human neutrophil-specific granule deficiency: a model
towards the role of neutrophil-specific granules in the evolution of

Bacterial nitric oxide synthase inhibits phagocytic cell NADPH
oxidase by interaction with neutrophils. J Biol Chem 1999;274:11253–
7.

[29] Houchos JT, Jones OT. The inhibition by diphenyleneiodonium and
its analogues of superoxide generation by macrophages. Biochem J

[30] Boyum A. Isolation of mononuclear cells and granulocytes from
human blood. Isolation of mononuclear cells by centrifugation, and
of granulocytes by centrifugation and sedimentation at 1 g. Scand J Clin Lab
Invest Suppl 1968;57:77–89.

[31] Fialkow L, Chan CK, Rotin D, Grinstein S, Downey GP. Activation of
the neutrophil–endothelial protein kinase signaling pathway in neutrophils.

activates Rac and Rho in human neutrophils: potential role in activation

[33] Chua Y, Solaki PA, Khosravi-Far R, Der CJ, Kelly K. The
inactivation of granulocyte-specific protein phosphatases by
MKP-1 and MKP-2 have unique substrate specificities and reduced activity

[34] Fialkow L, Chan CK, Rotin D, Grinstein S, Downey GP. Activation of
the neutrophil–endothelial protein kinase signaling pathway in neutrophils.

activates Rac and Rho in human neutrophils: potential role in activation


