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Reduction of Transition Metals by Human (THP-1) Monocytes is Enhanced by Activators of Protein Kinase C*

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Macrophages oxidize low-density lipoprotein (LDL) by enzymatic and non-enzymatic mechanisms; however, it is evident that macrophage reduction of transition metals can accelerate LDL oxidation *in vitro*, and possibly *in vivo*. Distinct cellular pathways contribute to this process, including trans-plasma membrane electron transport (TPMET), and production of free thiols or superoxide. Here, we explore the role of protein kinase C (PKC) in regulating transition metal reduction by each of these redox-active pathways, in human (THP-1) monocytes. We demonstrate that PKC agonists and/or inhibitors modulate reduction of transition metals by monocytes: both thiol-independent (direct) and thiol-dependent (indirect) pathways for transition metal reduction are enhanced by PKC activation, suggesting a potential strategy for therapeutic intervention.

Keywords: Atherogenesis, monocyte, oxidized low-density lipoprotein, protein kinase C, transition metals, thiols

Abbreviations: LDL, Low-density lipoprotein; TPMET, trans-plasma membrane electron transport; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); BPS, bathophenanthroline disulphonic acid; BCS, bathocuproinedisulphonic acid; LPS, lipopolysaccharide

INTRODUCTION

Accumulating evidence indicates that LDL is oxidized within the artery wall,[1] and that, once oxidized, it is profoundly pro-atherogenic; unlike native LDL, oxidized LDL enhances recruitment of monocytes,[2] elicits smooth muscle cell migration and proliferation[3] and generates cytokine-producing, lipid-laden macrophage foam cells.[4] Moreover, several cell types endogenous to arterial vessels oxidize LDL *in vitro*,[5–9] implying that cellular mechanisms are essential for conversion of LDL to an atherogenic particle *in vivo*. Both enzymatic and non-enzymatic mechanisms may be involved: reactive nitrogen species,[10,11] myeloperoxidase-generated reactive chlorine

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species,[12,13] 15-lipoxygenase-generated lipid hydroperoxides[14] and transition metals[15–17] have all been proposed as species contributing to development of human atherosclerotic lesions.

Redox-active free transition metals can be found within human atherosclerotic plaque;[15–17] moreover, physiologically relevant sources of transition metals, like ferritin,[18] haemin[19] or caeruloplasmin,[20] can enhance LDL oxidation *in vitro*. Indeed, the majority of cellular studies of LDL oxidation *in vitro* demonstrate dependence upon the presence of trace amounts of transition metals, in order to elicit the gross oxidative and structural changes conferring macrophage scavenger receptor recognition.[5–9] The pathways by which arterial cells, like macrophages, accelerate the oxidation of LDL in the presence of transition metals may, therefore, be important in understanding the aetiology of atherogenesis;[21] macrophages are quantitatively one of the most important cell types present during this process.[22]

Evidence indicates that more than one cellular mechanism is involved in the reduction of transition metals, and acceleration of LDL oxidation, by macrophages.[21,23–31] Production of cellular reductants, predominantly free thiols,[23–28] accelerates LDL oxidation in iron-containing Hams F10 culture medium. Importantly, this does not involve initiation of lipid peroxidation within the LDL particle;[29] rather, the thiol-dependent reduction of Fe^{3+} to Fe^{2+}(Equation (1)) catalyses the decomposition of low 'seeding' levels of lipid peroxides and thus is essentially propagative in nature (Equation (2)). This process involves the active uptake of L-cystine, via the x_c^- transporter,[26,31] cellular reduction, and subsequent release of free thiol (predominantly L-cysteine) into the culture medium.[26–28,31] Thiol 'recycling' occurs in activated human monocyte-derived macrophages (primary, and THP-1),[27,28] murine peritoneal macrophages[31] and rabbit endothelial cells;[26,31] cell types, like rat A10 smooth muscle cells, which do not exhibit this process, oxidize LDL at a much slower rate.[28]

$$RS^- + M^{n+} \rightarrow M^{(n-1)+} \quad (1)$$

$$LOOH + M^{(n-1)+} \rightarrow LO^\bullet + OH^- + M^{n+} \quad (2)$$

$$M^{n+} + e^- \text{ (cell derived)} \rightarrow M^{(n-1)+} \quad (3)$$

$$M^{n+} + O_2^{\bullet -} \leftrightarrow M^{(n-1)+} + O_2 \quad (4)$$

Nevertheless, transition-metal-dependent oxidation of LDL is still observed in both A10 smooth muscle cells,[28] and in human monocytes and macrophages incubated in the absence of L-cystine,[21,28,30] implying that thiol-independent reduction of transition metals occurs.[21,30] Recent reports demonstrated that macrophages reduce transition metals directly (Equation (3)), in part by trans-plasma membrane electron transport,[21] arguing that thiol export is not a strict requirement for cellular acceleration of LDL oxidation.[21,30] Direct trans-plasma membrane electron transport (TPMET) appears ubiquitous in mammalian cells,[32] uses NADH as an electron donor[33] and can mediate the reduction of extracellular copper and ferricyanide.[21,34] Alternatively, production of other (unidentified) cellular reductants, or of superoxide (Equation (4)) could reduce transition metals and accelerate LDL oxidation (Equation (2)).

Importantly, the cellular pathways which contribute to the reduction of transition metals are regulated by hormones,[34] inflammatory mediators[35,36] and growth factors.[37] Identification of a convergent signal transduction pathway by which these distinct mechanisms might be controlled during chronic inflammation could, therefore, be of therapeutic use. Previous studies have separately suggested TPMET,[38] superoxide production[39] and thiol output[35] can be enhanced by PKC agonists. Here, we have examined the role of protein kinase C (PKC) in regulating transition metal reduction by human (THP-1) monocytes. We confirm that both thiol-dependent and -independent pathways for transition metal reduction exist in PMA-differentiated THP-1 macrophages. In undifferentiated THP-1 monocytes,

direct (thiol-independent) transition metal reduction, presumably mediated via TPMET, can be enhanced by PKC agonists; this process appears largely independent of superoxide production via the respiratory burst elicited by PKC agonists. Data suggest thiol-dependent transition metal reduction can also be enhanced by PKC agonists; this pathway is induced during monocyte activation and can be blocked by PKC inhibitors. The results presented here indicate that PKC inhibitors may be useful in limiting transition metal redox cycling during inflammation.

MATERIALS AND METHODS

Materials Tissue culture medium and reagents, including customized Hams F10 formulations, were purchased from Gibco BRL (Paisley, Scotland). Tissue culture plastics were purchased from Falcon (Becton Dickinson, Oxford, England); all other chemicals were supplied by the Sigma Chemical Co. (Dorset, England).

Cell Culture Human (THP-1) monocytes were maintained exactly as described.[27,28] In experiments using phorbol ester-differentiated macrophages, THP-1 monocytes were seeded at $1–2 \times 10^6$ cells/well into 6- or 12-well tissue culture plates, and cultured in RPMI 1640 medium supplemented with glutamine (4 mM), penicillin/streptomycin (each 20 IU/ml), bovine foetal serum (10%, v/v), and phorbol 12-myristate 13-acetate (PMA, 160 nM). Macrophages were cultured for 5–7 days, renewing the medium at two day intervals.

In experiments using monocytes, cells were seeded at 2×10^6/well, into 12- or 24-well plates. For measurement of thiol production of [^{14}C]cystine uptake, monocytes were incubated in RPMI 1640 medium (above) and the appropriate concentrations of each agent to be tested. After incubation for 18 h, monocyte–macrophages were resuspended in Hams F10 nutrient mixtures; [^{14}C]cystine uptake was assessed after incubation for 1 h at 37°C, while the accumulation of free thiol was assessed after incubation for a further 8 h. Total cell numbers did not change during these incubations; non-adherent cells were returned to each respective well after resuspension in Hams F10 medium. In experiments where direct reduction of transition metals was measured, monocytes (2×10^6) were resuspended directly in Hams F10 medium (formulated without iron or cystine) containing the appropriate concentration of each test agent, and incubated for up to 3 h at 37°C.

Measurement of Superoxide Production Production of superoxide by THP-1 monocytes was measured as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c.[40] Briefly, monocytes (2×10^6) were incubated in Hams F10 medium (formulated without cystine or iron) containing ferricytochrome c (80 μM) in the presence or absence of SOD (10 μg/ml; 44 U/ml) and the concentrations of PKC agonists indicated in Figure legends. After 1 h at 37°C, cells were removed by centrifugation (10,000*g*) and cytochrome c reduction measured by absorbance at 550 nm ($\varepsilon = 21{,}000\ M^{-1}cm^{-1}$). Results are expressed as nanomoles superoxide produced/h/10^6 monocytes.

Measurement of Extracellular Thiols Accumulation of extracellular free thiol was determined using the thiol-specific reagent DTNB, exactly as described.[26–28] L-cysteine was used to construct a standard curve, linear from 0–125 μM.

Measurement of [^{14}C]Cystine Uptake Cystine uptake was measured essentially as described;[35] briefly, cells were incubated with 0.5 μCi [^{14}C]cystine/ml medium (final concentration 50 μM) for 2 min. The cells were then washed three times in ice-cold Dulbecco's phosphate-buffered saline (PBS), and the cell-associated radioactivity determined by scintillation counting.

Measurement of Transition Metal Reduction Reduction of iron, or copper, was assessed by formation of the Fe(II) · bathophenanthroline disulphonic acid ($\varepsilon 535\ nm = 22{,}522\ M^{-1}\ cm^{-1}$[21]) or Cu(I) · bathocuproine disulphonic acid ($\varepsilon 482\ nm = 9058\ M^{-1}\ cm^{-1}$[21]) complexes,

respectively. Cell-free reduction of transition metals was determined in Hams F10 medium containing copper sulphate (50 μM) and BCS (360 μM) or ferric citrate (50 μM) and BPS (360 μM) in the presence or absence of cysteine (0–100 μM) or PKC agonists/inhibitors. Cellular reduction of copper, in the presence or absence of extracellular thiols, was measured in Hams F10 medium (formulated without iron or cystine) containing $CuSO_4$ (50 μM) and BCS (125 μM), exactly as described;[21] reduction of iron(III) by macrophages was measured in the same medium containing Fe(III) citrate (50 μM) and BPS (360 μM).

Statistics All statistical tests were performed using the Student's *t*-test, as indicated in the legends to Figures and Tables; a statistical difference was reported when $p < 0.05$. All measurements were made in triplicate, or greater, within each experiment; numbers of independent experiments are indicated in the legends to Figures and Tables.

RESULTS

Thiol Dependent and -Independent Reduction of Iron and Copper by THP-1 Macrophages

Our previous studies clearly demonstrate that PMA-differentiated THP-1 macrophages produce extracellular free thiols, and exhibit thiol-dependent increases in LDL oxidation.[27,28] These cells, and human monocyte-derived macrophages,[21,28] also appear to exhibit thiol-independent oxidation of LDL; however, it was not clear whether THP-1 macrophages also possess the ability to reduce transition metals in the absence of thiol production. Macrophages (THP-1) were incubated in Hams F10 formulated without iron or L-cystine, and containing either copper(II) sulphate (50 μM) and bathocuproine disulphonic acid (BCS, 125 μM), (Figure 1A) or iron(III) citrate (50 μM) and bathophenanthroline disulphonic acid (BPS, 360 μM) (Figure 1B). One electron reduction of either Cu^{2+} or Fe^{3+}, as detected by formation of Cu(I) · BCS or Fe(II) · BPS complexes, occurs in the absence of thiol production (ensured by use of L-cystine free medium,[27]) and to a much greater extent than in the absence of cells. The origin of the cell-free reduction of copper and iron is not clear, but is presumably mediated by a component(s) of Hams F10 medium; in subsequent experiments, transition metal reduction in the absence of cells was subtracted from all samples. As expected, however, addition of L-cystine (100 μM) to the culture medium significantly enhances cellular reduction of transition metals, even during this short incubation (1 h).

One possible problem here is that chelation of cellular iron, and inhibition of TPMET, can occur following the direct addition of BPS to cultured cells;[41] while this should be minimized by the addition of Fe(III) citrate (50 μM),[42] it cannot be ruled out. In all other experiments, therefore, we use Cu^{2+} reduction to demonstrate direct transition metal reduction by macrophages,[21] and Fe^{3+} reduction to demonstrate indirect, thiol-dependent transition metal reduction by conditioned medium isolated from the cells (below).

These experiments established that thiol-dependent, and -independent pathways contribute to transition metal reduction in differentiated (THP-1) macrophages and suggested that activation of protein kinase C may be a key mechanism controlling both thiol-dependent and -independent pathways for transition metal reduction. We investigated this possibility, by treating THP-1 monocytes with PKC agonists, and measuring the reduction of copper or iron.

Direct (thiol-independent) Reduction of Copper(II) to Copper(I) by Human Monocytes

Direct reduction of transition metals in the absence of cellular thiol production is mediated, at least in part, by TPMET,[21] a system which can be regulated by growth factors, oncogenes, hormones and, importantly, by activation of

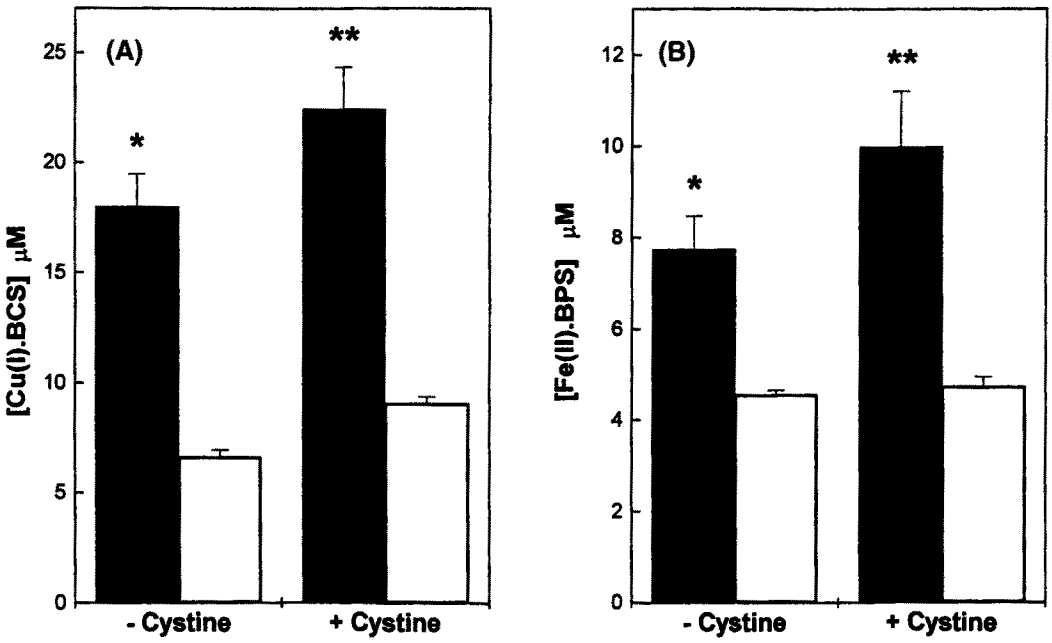

FIGURE 1 Human (THP-1) macrophages were differentiated by addition of PMA (160 nM) and cultured for 5–7 days (Methods). Transition metal reduction was detected by the formation of copper(I)·BCS (A) or iron(II)·BPS (B) complexes (Methods) during incubation (1 h) in the presence (closed bars) or absence (open bars) of THP-1 macrophages. Experiments were performed in Hams F10 medium, formulated without iron, and with or without L-cystine (100 μM). Values are the mean ± SD of nine replicate wells within the same experiment; *indicates values significantly ($p < 0.05$) different from the incubation in the absence of cells; **indicates values significantly ($p < 0.05$) different from incubations in the presence of cells, but the absence of L-cystine.

PKC.[32–34,36–38] In order to investigate the effect of PKC activation on direct transition metal reduction, THP-1 monocytes were incubated for 1 h in Hams F10 medium (without cystine or iron) containing $CuSO_4$ (50 μM) and BCS (125 μM), and in the presence or absence of PKC agonists PMA (160 nM), bryostatin-1 (100 nM) or thymeleatoxin (100 nM). We have previously measured thiol production by THP-1 macrophages incubated under similar conditions.[27,28] In the absence of L-cystine, very low levels of free thiol accumulate in the medium, albeit in a linear manner;[27] however, only 1.32 ± 0.104 μM free thiol could be measured after incubation for 24 h (0.055 nmol/ml/hr). This flux of free thiol is well below that required to mediate reduction of Fe(III) to Fe(II) in the assay system employed here (Methods) (see Figure 5B), even if allowance were made for the inevitable autooxidation of L-cysteine to L-cystine in the culture medium.[28] Garner *et al.*[21] have also discounted the possibility that leakage of free thiols is responsible for the direct reduction of copper by human monocyte-derived macrophages.

Reduction of Cu^{2+} to Cu^{+}, detected by BCS chelation,[21] occurred in the absence of PKC agonists, indicating that this pathway is not dependent upon PKC activation (Figure 2A). However, copper reduction was stimulated by a small (8.5 ± 4.8%, mean ± SD; $n = 3$), but significant ($p < 0.05$) extent by PMA (160 nM). More marked increases were observed following stimulation with bryostatin-1 (100 nM; 37 ± 2.5%; $n = 3$) or thymeleatoxin (100 nM; 61 ± 1.6%; $n = 3$). PKC agonists did not themselves alter the rate of copper reduction occurring in the absence of cells (data not shown). Enhanced copper reduction, following addition of bryostatin-1 (100 nM) or thymeleatoxin (100 nM), was observed at each time point examined (Figure 2B). Thus, direct

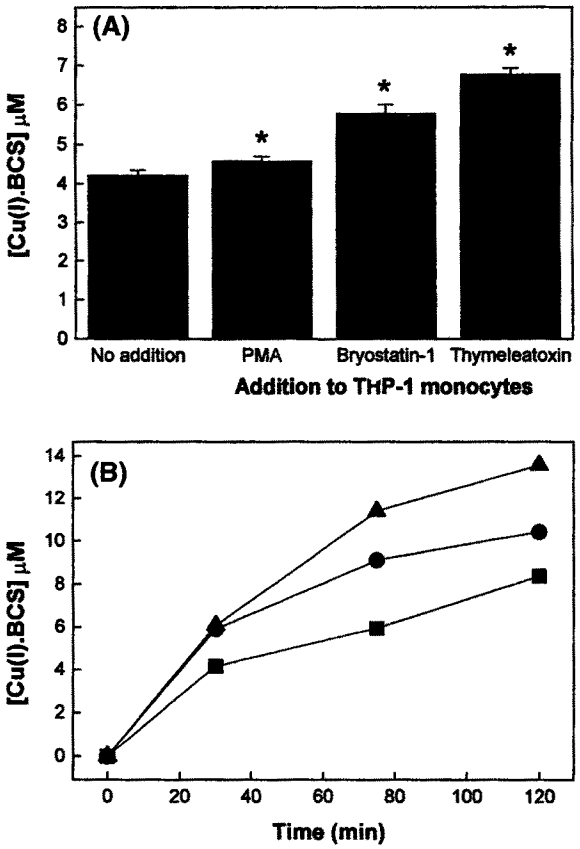

FIGURE 2 Reduction of copper(II) to copper(I) was measured during incubation of THP-1 monocytes in the absence (■) or presence of PMA (160 nM), bryostatin-1 (100 nM) (●) or thymeleatoxin (100 nM) (▲). Incubations proceeded for 1 h (A) or the indicated periods of time (B) in Hams F10 medium formulated without cystine or iron. Values in (A) are the mean ± SD of three independent experiments, following subtraction of the cell-free control; *indicates values significantly ($p < 0.05$) different from the control incubation. Values in (B) are from a single representative experiment, confirmed by one further experiment; errors from replicate wells fall within the symbol area and are not shown.

transition metal reduction is enhanced by, but is clearly not dependent upon, PKC activation.

Superoxide Production and Direct (thiol-independent) Reduction of Copper(II) to Copper(I)

It was possible that the enhanced transition metal reduction seen in the presence of PKC agonists was due to superoxide production during the respiratory burst elicited by these agents in monocytes.[39] We therefore investigated the contribution of superoxide to direct transition metal reduction in THP-1 monocytes. As expected, superoxide production was significantly ($p < 0.05$) enhanced by the addition of PMA (160 nM), bryostatin-1 (100 nM) or thymeleatoxin (100 nM) to THP-1 monocytes (Figure 3A). Addition of superoxide dismutase (SOD, 10 μg/ml) to the culture medium completely blocked superoxide-dependent reduction of cytochrome c. However, it is evident from Figure 2A that copper reduction can proceed effectively in the absence of superoxide production (Figure 3A), and addition of SOD (0.1–1 μg/ml) did not significantly inhibit the reduction of copper in control incubations (Figure 3B). Moreover, SOD was not an effective inhibitor of copper reduction in cells stimulated with PKC agonists (Figure 3B and C); in three independent experiments, reduction of Cu^{2+} to Cu^{+} by cells treated with thymeleatoxin (100 nM) was decreased by only 35 ± 2.3% (mean ± SD; $p < 0.05$) by the presence of SOD (10 μg/ml) and remained significantly higher than the control incubation (Figure 3C). It appears that generation of superoxide plays a relatively minor role in the thiol-independent reduction of transition metals by THP-1 monocytes, even in the presence of PKC agonists.

Activation of PKC enhances Thiol 'Recycling' by THP-1 Monocytes

Unlike the regulation of TPMET[38] and superoxide release,[39] the effect of PKC activation on thiol 'recycling' in human macrophages has not been fully characterized. We therefore explored the dependency of this pathway on PKC activation, using PKC agonists and inhibitors; the inflammatory mediator, bacterial lipopolysaccharide (LPS), was also employed to confirm the physiological relevance of these findings.[35]

Thiol release was significantly increased in response to PMA, bacterial lipopolysaccharide (LPS) (Figure 4A), bryostatin-1 (100 nM) and

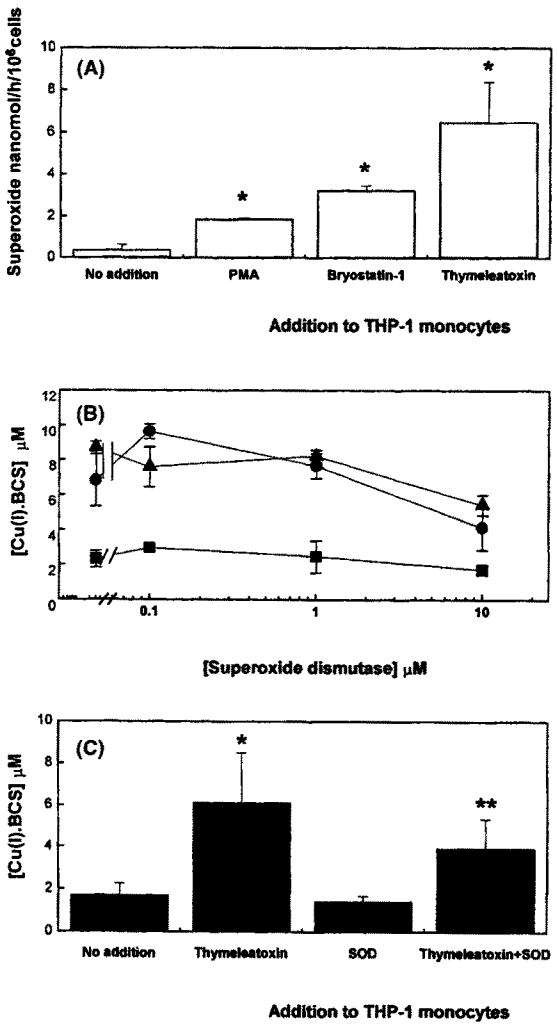

FIGURE 3 Superoxide production by THP-1 monocytes was measured (Methods) during incubations (1 h) in the absence or presence of PMA (160 nM), bryostatin-1 (100 nM) or thymeleatoxin (100 nM) (A). Values are means ± SD of three independent experiments; *indicates values significantly ($p < 0.05$) different from the control incubation. Cellular reduction of copper(II) to copper(I) was measured (Methods) following incubation in the absence (■) or presence of bryostatin-1 (100 nM) (●) or thymeleatoxin (100 nM) (▲), in medium containing the indicated concentrations of superoxide dismutase. (B). Values are the means ± SD of triplicate wells within a single experiment. Results from (B) were confirmed in three independent experiments (mean ± SD) (C); *indicates values significantly different from the control incubation; **indicates values significantly different from incubations in the presence of SOD (10 μg/ml). All experiments were performed in Hams F10 medium formulated without cystine or iron; all values are corrected for the cell-free reduction of transition metals which occurs in Hams F10 medium.

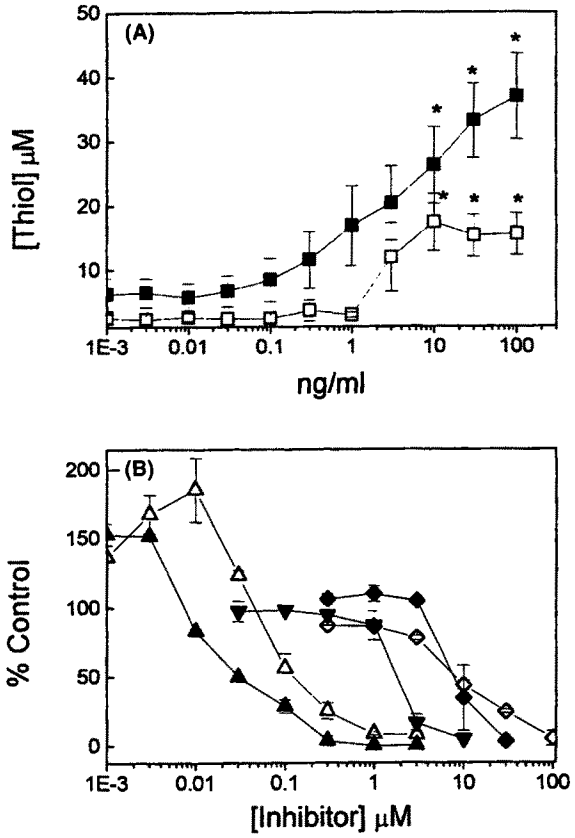

FIGURE 4 Release of free thiol from THP-1 monocytes was measured following preincubation (18 h) in RPMI medium (Methods) containing PMA (□) or lipopolysaccharide (■); monocytes were then resuspended in Hams F10 medium, formulated without iron but containing L-cystine (100 μM) and incubated for a further 8 h before the thiol content of the medium was determined (Methods) (A). Values are the mean ± SEM of four independent experiments; *indicates values significantly different ($p < 0.05$) from the control incubation in the presence of cells. Inhibition of thiol release, induced by either LPS (10 ng/ml) (closed symbols) or PMA (32 nM) (open symbols) (B), was achieved by simultaneous preincubation (18 h) in the presence of staurosporine (up-triangles), Ro-31-8220 (down-triangles) or chelerythrine chloride (diamonds) at the concentrations indicated. Values are mean ± ranges of two independent experiments.

thymeleatoxin (100 nM) (Table I). Export of thiols was significantly enhanced ($p < 0.05$) at concentrations of 3.0 ng PMA/ml; however, at higher concentrations (100 ng/ml; 160 nM), thiol release was not consistently optimal, explaining the differences between PKC agonists observed in Figures 4 and 5. Induction of thiol release by PMA

TABLE I Thiol release and [^{14}C]cystine uptake by THP-1 monocytes

Condition	Thiol release (μM)	[^{14}C]cystine uptake (% control)
No addition	6.06 ± 1.04 (10)	100%
PMA (32 nM)	32.8 ± 3.08 (5)*	206.6 ± 41.7 (4)*
PMA (32 nM) + Staurosporine (1 μM)	0.667 ± 0.13 (2)	80.7 ± 17.4 (4)
Lipopolysaccharide (10 ng/ml)	33.8 ± 2.12 (6)*	158.5 ± 18.4 (4)*
LPS (10 ng/ml) + Staurosporine (1 μM)	0 ± 0.0 (2)	72.6 ± 19.7 (4)
Staurosporine (1 μM)	n.d.	70.7 ± 24.7 (4)
Bryostatin-1 (100 nM)	29.7 ± 4.34 (7)*	233.3 ± 33.8 (5)*
Thymeleatoxin (100 nM)	38.1 ± 1.78 (2)	229.9 ± 56.3 (5)*

Monocytes (THP-1) were incubated in RPMI 1640 medium, containing foetal bovine serum (10%, v/v) and the indicated concentrations of each agent, for 18 h. The cells were then resuspended in Hams F10 medium and assayed for uptake of [^{14}C]cystine, or incubated for a further 8 h so that thiol accumulation could be measured (Methods). Values are means ± SEM, or means ± ranges for the number of independent experiments shown in parentheses; the absolute value for [^{14}C]cystine uptake was 21.2 ± 2.64 pmol/min/well (mean ± SEM, $n = 5$). *Values differing significantly ($p < 0.05$) from the control (no addition) incubation. n.d.: not determined.

or bacterial LPS was reversed by the addition of PKC inhibitors: staurosporine, chelerythrine chloride or Ro-31-8220 (Figure 4B). It is evident that staurosporine exerts biphasic effects upon thiol release; this drug has previously been shown to exert paradoxical agonistic and antagonistic properties.[43] Since staurosporine can also inhibit other serine/threonine and tyrosine kinases, we additionally employed chelerythrine chloride and Ro-31-8220, which do not inhibit tyrosine kinases, calcium/calmodulin-dependent protein kinase or protein kinase A, at the concentrations used here.[44,45] Addition of PKC agonists, or LPS (10 ng/ml)[35] were, as expected, similarly associated with increases in [^{14}C]cystine transport; these increases could also be prevented by PKC inhibitors (Table I).

Thiol-dependent Reduction of Fe^{3+} to Fe^{2+} by Conditioned Medium from THP-1 Monocytes

Release of thiols from THP-1 monocytes is enhanced by PKC agonists, suggesting that activation of PKC should also stimulate thiol-dependent reduction of transition metals. In order to demonstrate this, THP-1 monocytes were preincubated (18 h) in the presence or absence of PMA (160 nM), bryostatin-1 (100 nM) or thymeleatoxin (100 nM). The cells were then resuspended in Hams F10 medium, formulated with L-cystine (100 μM) but without iron, and incubated for a further 8 h. This experimental protocol allowed the accumulation of extracellular free thiol in the absence of, but following stimulation by, PKC agonists. The medium was centrifuged (10,000g) to remove cellular debris, before addition of Fe(III)citrate (50 μM) and BPS (360 μM). Medium from control monocytes reduced Fe^{3+} to Fe^{2+} (Figure 5A); however, this process was significantly ($p < 0.05$) enhanced by prior incubation with PMA (19 ± 3.2%; mean ± SD, $n = 3$), bryostatin-1 (53 ± 2.3%; $n = 3$) or thymeleatoxin (76.2 ± 8.5%; $n = 3$).

Importantly, increases in iron(III) reduction were highly correlated ($R = 0.99$) with the accumulation of extracellular free thiols produced by THP-1 monocytes (Figure 5B). Moreover, reduction of iron by exogenous cysteine (inset) gave similar results to those mediated by endogenously generated free thiols; around 5.5 nmole of free thiol were apparently required to generate 1 nmole of Fe(II) · BPS complex (Figure 5B), reflecting the ionization of RSH to RS required for reaction with Fe^{3+} (Equation (1)).

DISCUSSION

Reduction of transition metals, via both thiol-dependent and -independent routes, is one

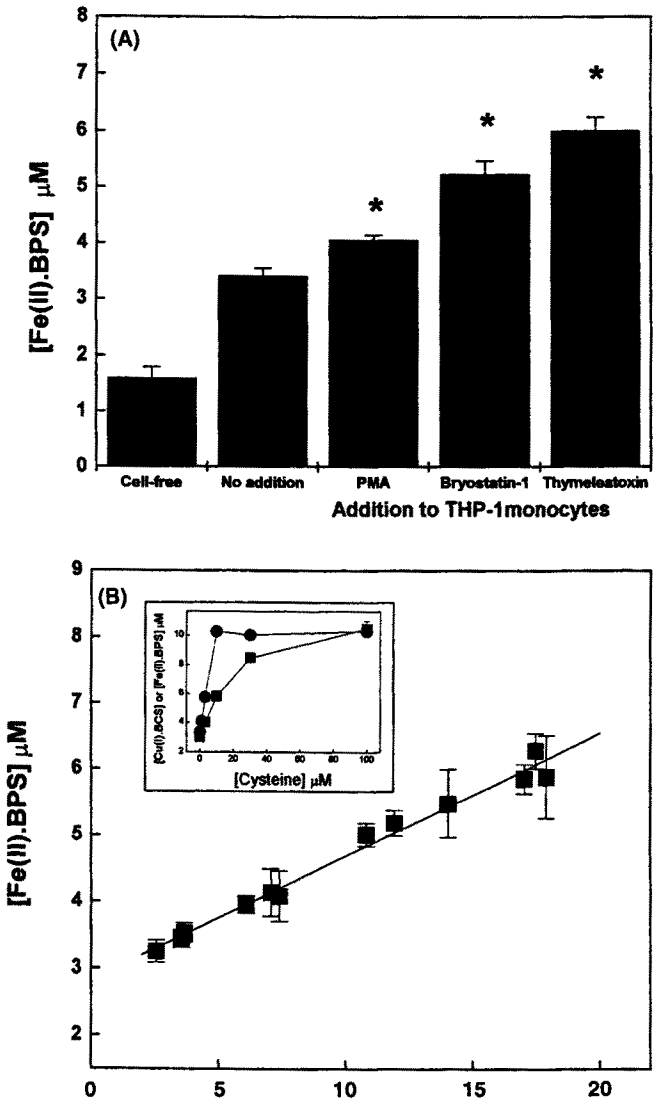

FIGURE 5 Reduction of iron(III) to iron(II) by conditioned media (Methods) from THP-1 monocytes was performed following preincubation (18 h) in RPMI medium (Methods) containing PMA (160 nM) bryostatin-1 (100 nM) or thymeleatoxin (100 nM) (A). Monocytes were then resuspended in Hams F10 medium, formulated without iron but with L-cystine (100 μM) and incubated for a further 8 h to allow thiols to accumulate. Values in (A) are means ± SD of three independent experiments; *indicates values significantly differing from the control incubation in the presence of cells. Values in (B) are the means ± SD of triplicate wells within each experiment, expressed relative to the free thiol content of the conditioned media. Reduction of iron(III) to iron(II), and copper(II) to copper(I), by exogenously added L-cysteine is shown in inset; values are the mean ± SD of three independent experiments; however, error bars fall within the area of the symbol and are not shown.

mechanism by which human macrophages can accelerate the oxidation of LDL.[21,23–31] Reduced transition metals enhance the oxidation of LDL by increasing the rate of decomposition of lipid hydroperoxides within the lipoprotein particle; reduced transition metals, such as Fe^{2+} or Cu^{+}, react much faster with lipid hydroperoxides, thereby accelerating the oxidative process (Equations 1–4). Thus, cellular mechanisms which enhance transition metal reduction exert pro-oxidant effects upon the oxidation of LDL in transition-metal-containing medium.[21,23–31]

Further, since redox-active transition metals are reportedly present within human atherosclerotic lesions,[15–17] these mechanisms may play a key role in propagating and exacerbating oxidative processes within the artery wall. Consistent with this hypothesis is our finding that macrophage-reduction of transition metals, via both transition-metal-dependent and -independent mechanisms, is enhanced under pro-inflammatory conditions.

Basal levels of cellular transition metal reduction were evident, arguing that activation of PKC is not obligatory for either pathway in THP-1 monocytes (Figures 2A and 5A). Both thiol-dependent, and -independent pathways of transition metal reduction were also active in phorbol ester-differentiated THP-1 macrophages (Figure 1); these cells were no longer responsive to treatment with PKC agonists (data not shown). However, PKC agonists did enhance the ability of THP-1 monocytes to mediate both thiol-independent (direct) and thiol-dependent (indirect) reduction of transition metals. The phorbol ester PMA, which activates all PKC isoforms except PKC-ζ,[46] enhanced the direct reduction of transition metals (Figure 2A), but was more effective in stimulating thiol release and cystine uptake by THP-1 monocyte/macrophages (Table I, Figure 4A). Since phorbol esters can exert PKC-independent effects,[47] we also used a non-phorbol ester PKC agonist, the macrocyclic lactone bryostatin-1, to activate PKC.[46] This agent, which has been shown to activate PKC isoforms α, β, δ and ε,[46,48] also increased thiol output, superoxide production and direct transition metal reduction (Figures 2A, 3A and 5A; Table I). However, the efficacy of thymeleatoxin, a second stage tumour-promoting agent which exerts (limited) selectivity against differing PKC isoforms, activating α-, β1- and γ-, but not δ- and ε-, isotypes,[48] implies that calcium-dependent PKC isoforms may be involved in enhancing transition metal reduction in THP-1 monocytes. Reasons for the differing potencies of the PKC agonists employed have not been specifically addressed in this study. However, divergent biological actions have been observed for PMA, bryostatin-1, and thymeleatoxin in a number of different cell types,[48,49] and variously attributed to the differential activation and/or down-regulation of PKC isotorms in response to these agents.[48,49]

Thiol-independent reduction of transition metals, which is enhanced by the presence of PKC activators, could be mediated by release of superoxide or an alternate reductant, or directly by TPMET by THP-1 monocytes. However, it seems unlikely that generation of superoxide contributes significantly to the thiol-independent reduction of copper(II) to copper(I) by THP-1 monocytes (Figure 2). Copper reduction can proceed in the absence of superoxide production (Figures 2A and 3A), and superoxide dismutase is not an effective inhibitor of copper reduction in cells stimulated with PKC agonists (Figure 3B and C).

Although it is possible that alternate reductants could be released, and contribute to transition metal reduction by THP-1 monocytes, a systematic search for such species by Garner *et al.* suggested that free thiols were the sole reductant released, at least under basal conditions.[21] Direct reduction of copper(II) to copper(I) by monocyte/macrophages is thought to be mediated via TPMET,[21] which is known to be regulated by activation of PKC.[38] Our data confirm that THP-1 monocytes exhibit direct (thiol-independent) reduction of copper(II) to copper(I)[21] and appear to suggest that PKC-enhanced TPMET is an important factor determining the rate of transition metal reduction by monocyte–macrophages.

Thiol-dependent reduction of transition metals, specifically iron(III) to iron(II), is enhanced by PKC agonists (Figure 5). Both thiol output, and L-cystine uptake are enhanced by incubation with PKC agonists; moreoever, these effects can be blocked by the addition of PKC inhibitors (Figure 4, Table I). The effect of the inflammatory mediator, lipopolysaccharide, which is known to activate both thiol production[31,35,50] and cystine uptake[35] by macrophages, also appears dependent upon PKC activation (Figure 4B).

Conditioned medium, isolated from THP-1 monocytes incubated in the presence or absence of phorbol ester, bryostatin-1 or thymeleatoxin, contained differing concentrations of free thiols, which correlated closely with the ability of these media to reduce iron(III) to iron(II) (Figure 5B). Thus, the presence of PKC agonists enhances 'redox-cycling' of L-cystine and L-cysteine across the plasma membrane: macrophages release extracellular L-cysteine, which ionizes, reduces iron(III) to iron(II), and is thereby autooxidized to L-cystine,[28] which can then be taken up again for yet another 'cycle'.

We have demonstrated PKC agonists and/or inhibitors enhance both thiol-dependent and -independent pathways of transition metal reduction by human (THP-1) monocytes. However, this is clearly not the only signal transduction pathway involved in their regulation. Parallel increases in transition-metal-reducing pathways have also been noted following incubation with insulin; insulin increases ferricyanide reduction by HeLa cells[34] and increases thiol output by human macrophages.[51] In contrast, tumour necrosis factor-α (TNF-α) inhibits TPMET,[36] but strongly enhances production of free thiols by macrophages,[31,35,50] arguing that these pathways can be independently regulated under certain conditions. Thus, although differing pathways of transition metal reduction may be invoked, both insulin and TNF-α should increase LDL oxidation by macrophages, and indeed, this has proven to be the case.[31,51,52] Thus, it is apparent that both thiol-dependent and -independent transition metal reduction should be assessed when attempting to predict the effects of hormones, or inflammatory mediators on macrophage oxidation of LDL in transition-metal-containing media.

In summary, it appears that activation of PKC enhances two differing pathways of transition metal reduction by human macrophages, potentially contributing to the accelerated oxidation of LDL which may occur under these conditions.[53,54] Regulation of PKC activity, therefore, indicates a possible strategy by which these potentially pro-atherogenic processes may be controlled during the chronic inflammation which characterizes development of atherosclerotic lesions within the vessel wall.

Acknowledgments

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