

Research Article

Oxaprozin-Induced Apoptosis on CD40 Ligand-Treated Human Primary Monocytes Is Associated with the Modulation of Defined Intracellular Pathways

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Received 16 February 2009; Revised 2 June 2009; Accepted 17 June 2009

Recommended by Mostafa Z. Badr

The modulation of CD40L activity might represent a promising therapeutic target to reduce monocyte inflammatory functions in chronic diseases, such as rheumatoid arthritis. In the present study, we investigated the possible influence of nonsteroidal anti-inflammatory drugs (NSAIDs) on CD40L-induced monocyte survival. Monocytes were isolated from buffy coats by using Ficoll-Percoll gradients. Monocyte apoptosis was evaluated by fluorescence microscopy on cytopreps stained with acridine orange or using flow cytometry analysis of Annexin-V and Propidium Iodide staining. Akt and NF- κ B activation was assessed using western blot. Caspase 3 activity was determined spectrophotometrically. Among different NSAIDs, only oxaprozin dose-dependently increased apoptosis of CD40L-treated monocytes. Oxaprozin pro-apoptotic activity was associated with the inhibition of CD40L-triggered Akt and NF- κ B phosphorylation and the activation of caspase 3. In conclusion, our data suggest that oxaprozin-induced apoptosis in CD40L-treated human monocytes is associated with previously unknown cyclooxygenase (COX)-independent pathways. These intracellular proteins might be promising pharmacological targets to increase apoptosis in CD40L-treated monocytes.

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1. Introduction

Human monocytes are central players in the pathogenesis of chronic inflammatory disorders, such as atherosclerosis and rheumatoid arthritis [1–3]. Monocytes are immune cells circulating in the blood stream, which are recruited to inflammatory sites for the immune response [2, 4]. Once resident in inflamed tissues, they can differentiate into several cell types, such as macrophages, dendritic cells, and also osteoclast-like cells [5–7] contributing to further increase inflammation. In this context, the induction of monocyte apoptosis with the consequent reduction of inflammatory cell infiltrates has to be considered as a crucial target to reduce inflammatory disease persistence. CD40 ligand

(CD40L) is a crucial mediator in monocyte functions and differentiation [8]. Thus, the modulation of CD40L-induced proinflammatory activities could represent a very promising therapeutic strategy to reduce monocyte-mediated tissue infiltration and injury. In the present article, we planned to investigate the possible influence of nonsteroidal anti-inflammatory drugs (NSAIDs) on CD40L-treated monocyte apoptosis [9, 10]. In particular, we focused on oxaprozin, an achiral oxazole-propionic acid derivative (4,5-diphenyl-2-oxazolepropionic acid) that has been recently shown to modulate monocyte survival in inflammatory microenvironments [11]. In order to identify the possible molecular mechanisms involved in, we studied the role of different intracellular mediators, such as cyclooxygenase

(COX), phosphoinositol-3 kinase (PI3K)/Akt, extracellular signal-regulated kinase (ERK) 1/2, p38 mitogen activated kinase (MAPK), nuclear factor kappaB (NF- κ B), and caspase 3.

2. Materials and Methods

2.1. Human Monocyte Isolation. Human monocytes were isolated from buffy coats, obtained from healthy volunteers, under a protocol approved by the local Ethics Committee. All donors provided written, informed consent to the procedure and use of the cells. After centrifugation on a Ficoll-Hypaque density gradient, mononuclear cells were collected from the interface and washed with PBS. Monocytes were then purified from the upper interface of a hypotonic Percoll density gradient (1.129 g/mL). Purified monocytes were resuspended in RPMI 1640 medium supplemented with 1% (v/v) heat-inactivated fetal calf serum (FCS) and 500 ng/mL polymyxin B, as previously described [12]. At the end of purification, viability of monocytes was more than 98%, as determined by ethidium bromide-fluoresceine diacetate assay (from Sigma-Aldrich S.r.l., Milano, Italy). Monocyte purity was at least 90%, as assessed by flow cytometric analysis (staining with FITC-conjugated antihuman CD14 antibody (Ab), from BD Pharmingen, Franklin Lakes, NJ) and nonspecific esterase staining.

2.2. Assessment of Monocyte Apoptosis. Purified monocytes were resuspended at 10^6 /mL and cultured for 48 hours in culture medium containing RPMI 1640 with 25 mM HEPES (Irvine Scientific, Santa Ana, CA), 500 ng/mL polymixin B (Sigma-Aldrich), and 1% heat-inactivated FCS (ICN Biomedicals s.r.l., Milano, Italy) in the presence or absence of 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer (both from Alexis Biochemicals, Carlsbad, CA). In selective experiments, cells were cultured in the presence or absence of 50 μ M PD98059 (MEK inhibitor, a kinase activating ERK 1/2, from Biomol Research Laboratories, Inc., Plymouth Meeting, PA), 1 μ M SB203580 (p38 MAPK inhibitor, from Biomol Research Laboratories, Inc.), 50 μ M LY294002 (PI3K inhibitor, from Sigma-Aldrich), 20 μ M SN-50 (NF- κ B inhibitor, from Calbiochem, San Diego, CA), 50 μ M Ac-DEVD-CHO (caspase 3 inhibitor, from Bachem AG, Bubendorf, Switzerland), different doses (5, 10, 50, 100 μ M) of oxaprozin (Helsinn Healthcare SA, Lugano, Switzerland), 100 μ M ibuprofen (Sigma-Aldrich), 100 μ M indomethacin (Sigma-Aldrich), or 100 μ M naproxene (Sigma-Aldrich). Percentages of apoptotic cells were measured by both fluorescence microscope (staining with acridine orange from Sigma-Aldrich and Annexin V-FITC Kit, from MBL International, Woburn, MA) and flow cytometer (by using Propidium Iodide Kit, from MBL International). Immunofluorescence analysis of Annexin V and Propidium iodide binding was performed by following the manufacturer's instructions with minor changes, as previously described [13].

2.3. Immunoblot Analysis. 10^7 cells/mL monocytes were preincubated in the presence or absence of 100 μ M oxaprozin

for 1 hour in a humidified atmosphere 5% CO₂ at 37°C and then, without washing, stimulated in the presence or absence of 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer, for 15 minutes. The reaction was stopped on ice, and cells were centrifuged at 4°C to remove culture supernatants. Total protein was extracted in lysis buffer containing 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 1% Nonidet P-40, 10% glycerol, 1 mM PMSE, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, and 0.5 mM Na₃VO₄. Proteins (40 μ g) were electrophoresed through polyacrylamide-SDS gels and transferred by electroblotting onto nitrocellulose membranes, as previously [14]. Membranes were blocked for 1 hour in 5% (wt/vol) nonfat milk and then stained with monoclonal antihuman phosphorylated (p)-p65 NF κ B, monoclonal antihuman p-Akt (both from Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal antihuman p-p38 MAPK, or monoclonal antihuman p-ERK 1/2 Abs (both from R&D System, Minneapolis, MN).

2.4. Caspase 3 Activity. Caspase 3 activity in the presence or absence of 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer and 100 μ M oxaprozin was performed as previously described [15]. The enzymatic activity was spectrophotometrically determined (Titertek TwinReader Plus, Flow Lab, Ltd, Irvine, Scotland) for 60 minutes at 405 nm assuming an extinction coefficient of 8.8×10^3 M⁻¹ cm⁻¹.

2.5. Statistical Analysis. Statistical analysis was performed by using one-way ANOVA with Bonferroni's posttest (GraphPad InStat version 3.05 for Windows XP, GraphPad Software, San Diego, CA). All data were expressed as mean \pm SEM. Differences were accepted as significant when $P < .05$.

3. Results

3.1. Oxaprozin Increases Apoptosis of CD40L-Treated Monocytes. NSAIDs have been previously shown to modulate monocyte functions and monocytic cell survival [16, 17]. On the other hand, CD40 triggering has been already shown to enhance monocyte survival [9]. Confirming these data, Figure 1(a) shows that 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer induced a significant ($P < .05$) reduction of monocyte apoptosis in comparison with control medium. Oxaprozin dose dependently increased CD40L-treated monocyte apoptosis. 100 μ M oxaprozin induced the strongest proapoptotic effect. Oxaprozin-mediated activity was also confirmed using Annexin V and Propidium Iodide assays, assessed by fluorescence microscope and flow cytometry (Figure 1(b)). No significant effect on cell apoptosis/death was observed in untreated cells in the presence of different concentrations of oxaprozin (data not shown).

3.2. Oxaprozin Proapoptotic Effect Is Independent of COX Inhibition. To assess the possible role of COX in oxaprozin-mediated proapoptotic activity, we studied other NSAIDs, such as ibuprofen, indomethacin or naproxen. To inhibit COX, we used these drugs at higher concentrations (100 μ M) than their previously reported IC₅₀ for COX-1 and -2 in

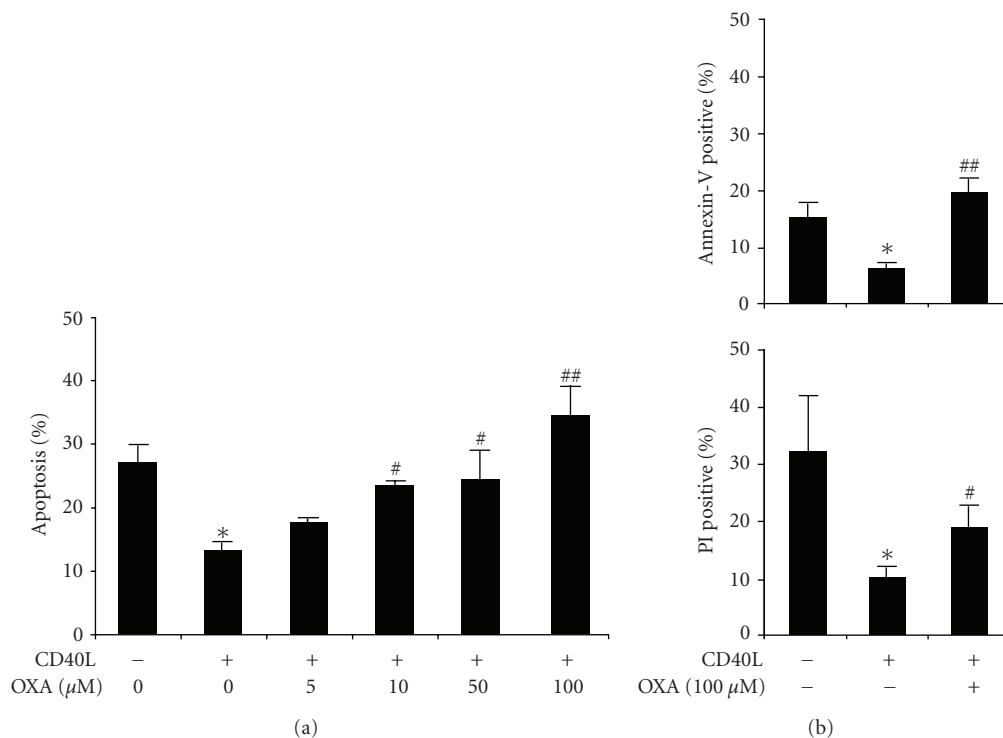


FIGURE 1: Oxaprozin increases apoptosis in CD40L-treated monocytes. Monocytes were incubated for 48 hours with control medium or 200 ng/mL CD40L plus 1 $\mu\text{g}/\text{mL}$ CD40L enhancer in the presence or absence of different doses (0, 5, 10, 50, 100 μM) of oxaprozin. (a) Assessment of apoptosis by acridine-orange-stained slides under fluorescence microscopy. Data are expressed as percentages of apoptotic cells on total number of cells counted (mean \pm SEM, $n=3$; apoptosis in absence versus in presence of CD40L: * $P<.05$; apoptosis in presence of CD40L versus CD40L plus 10 or 50 μM oxaprozin: # $P<.05$; apoptosis in presence of CD40L versus CD40L plus 100 μM oxaprozin: ## $P<.01$). (b) Assessment of apoptosis by using Annexin V and Propidium Iodide (PI) binding assay. (mean \pm SEM, $n=4$; apoptosis in absence versus in presence of CD40L: * $P<.05$; apoptosis in presence of CD40L versus CD40L plus 100 μM oxaprozin: # $P<.05$ and ## $P<.01$).

intact cells [18, 19]. 100 μM oxaprozin significantly increased CD40L-treated monocyte apoptosis. Conversely, treatments with ibuprofen, indomethacin, or naproxene did not induce any effect (Figure 2(a)). These data were also confirmed using Annexin V and Propidium Iodide assay (Figure 2(b)). No significant effect on cell apoptosis/death was observed in untreated cells in the presence of different NSAIDs at 100 μM (data not shown).

3.3. Oxaprozin Proapoptotic Activity Is Associated with the Inhibition of CD40L-Triggered Akt and NF- κB Phosphorylation.

In order to identify the intracellular pathway involved in oxaprozin-mediated proapoptotic activity, we investigated its possible modulatory effect on CD40L-triggered survival pathways. Figure 3(a) shows that CD40L-mediated reduction of monocyte apoptosis was partially reversed by pretreatment with LY294002 (PI3K inhibitor), PD98059 (MEK 1/2 inhibitor, a kinase activating ERK 1/2), SB203580 (p38 MAPK inhibitor), or SN-50 (NF- κB inhibitor). In particular, the increase of monocyte apoptosis observed in presence of only one of each selective inhibitor suggesting that all signaling pathway activation is required for CD40L-mediated antiapoptotic effects. Annexin V and Propidium Iodide assays confirmed apoptosis levels morphologi-

cally assessed (Figure 3(b)). Oxaprozin treatment inhibited CD40L-induced Akt and NF- κB (p65) phosphorylation (Figure 3(c)). Conversely, a slight increase of ERK 1/2 and p38 MAPK phosphorylation was observed in the presence of oxaprozin in CD40L-simulated monocytes. No significant activation/inhibition on protein phosphorylation in comparison with control medium was observed in the presence of oxaprozin alone (data not shown). These data suggest that oxaprozin treatment is associated with the inhibition of Akt and NF- κB phosphorylation in CD40L-treated monocytes. Although crucial in CD40L-induced monocyte survival, ERK 1/2 and p38 MAPK are not involved in oxaprozin-mediated proapoptotic effects.

3.4. Oxaprozin Proapoptotic Activity Is Associated with Caspase 3 Activation.

We also investigated oxaprozin activity on CD40L-mediated caspase 3 regulation, as a well-known apoptotic pathway [20, 21]. According to apoptosis experiments, oxaprozin treatment was associated with a significant increase of caspase 3 activity in CD40L-treated monocytes (Figure 4(a)). To further confirm the involvement of caspase 3 in oxaprozin-mediated proapoptotic activity, treatment with caspase 3 inhibitor blocked oxaprozin-induced apoptosis in CD40L-treated monocytes (Figure 4(b)).

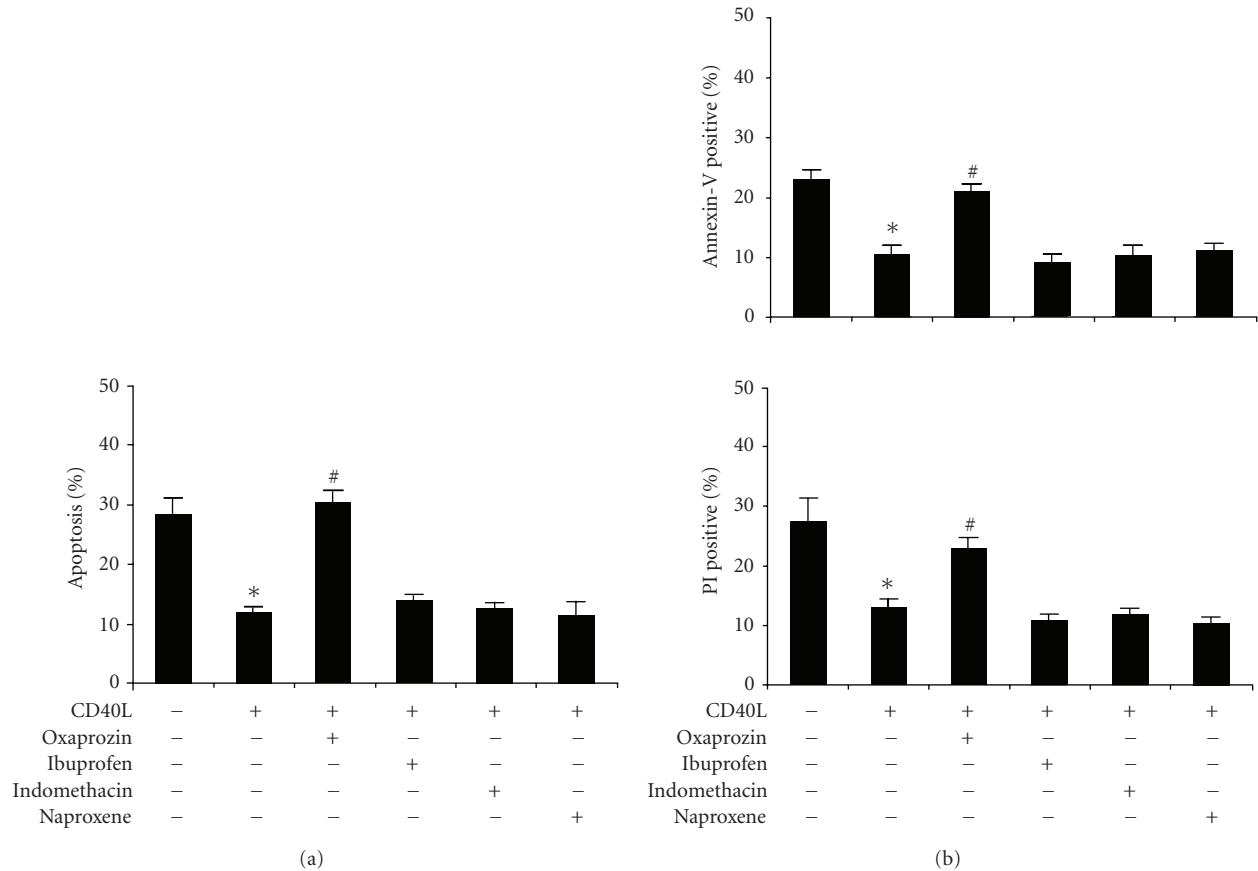


FIGURE 2: Oxaprozin-induced apoptosis is independent on COX inhibition. Monocytes were incubated for 48 hours with control medium or 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer in the presence or absence of 100 μ M oxaprozin, 100 μ M ibuprofen, 100 μ M indomethacin, or 100 μ M naproxene. (a) Assessment of apoptosis by acridine-orange-stained slides under fluorescence microscopy. Data are express, as percentages of apoptotic cells on total number of cells counted (mean \pm SEM, $n=4$; apoptosis in absence versus in presence of CD40L: * $P<.05$; apoptosis in presence of CD40L versus CD40L plus oxaprozin: # $P<.05$; apoptosis in presence of CD40L versus CD40L plus other NSAIDs: N.S.). (b) Assessment of apoptosis by using Annexin V and Propidium Iodide (PI) binding assay (mean \pm SEM, $n=3$; apoptosis in absence versus in presence of CD40L: * $P<.05$; apoptosis in presence of CD40L versus CD40L plus oxaprozin: # $P<.05$; apoptosis in presence of CD40L versus CD40L plus other NSAIDs: N.S.).

4. Discussion

The induction of monocyte apoptosis rather than necrosis might represent a crucial approach to chronic inflammatory diseases, such as rheumatoid arthritis and atherosclerosis. In fact, in contrast to cells undergoing necrosis, the contents of those undergoing apoptosis are not released but cleared as apoptotic bodies by phagocytosis. On the other hand, necrotic cell death promotes inflammation, while apoptotic cell death results in an anti-inflammatory condition. Therefore, immune cell apoptosis is actually considered as a very promising anti-inflammatory therapeutic strategy [22]. On the other hand, CD40L represents one of the most important mediators in inflammatory processes modulating both infections and chronic diseases, including atherosclerosis, rheumatoid arthritis and transplantation [23–28]. CD40L has been already shown to prolong monocyte survival [9]. CD40 is also present on B cell, dendritic cell, monocyte, macrophage, mast cell, fibroblast, and endothelial cell membrane and regulates

several immune functions, such as B cell response, antigen presenting cell activity, monocyte migration, and survival [29–31]. These activities resulted from the binding with two forms of CD40L. The first form is expressed on activated T and other immune cell membrane (CD154), while the second one is a soluble form, also called soluble CD40 ligand (sCD40L) [31]. Thus, given the pivotal role of monocytes and sCD40L in inflammatory diseases, we investigated the possible effect of the NSAIDs on CD40L-induced monocyte survival [32]. Among NSAIDs (drugs known for their potent COX inhibitory activity), only oxaprozin dose dependently increases apoptosis in CD40L-treated monocytes. This effect was observed at physiological concentrations achievable in inflamed tissues [33]. These data suggest that a possible molecular mechanism independent of COX inhibition might be associated with oxaprozin proapoptotic effect. To inhibit COX, we used NSAIDs at higher concentrations (100 μ M) than their previously reported IC_{50} for COX-1 and -2 in intact cells [19]. In particular, IC_{50} of oxaprozin for human platelet COX-1 and for human interleukin-1 β stimulated

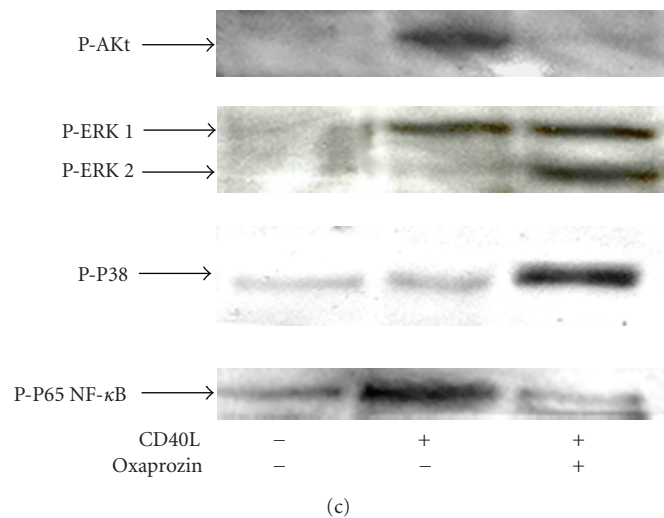
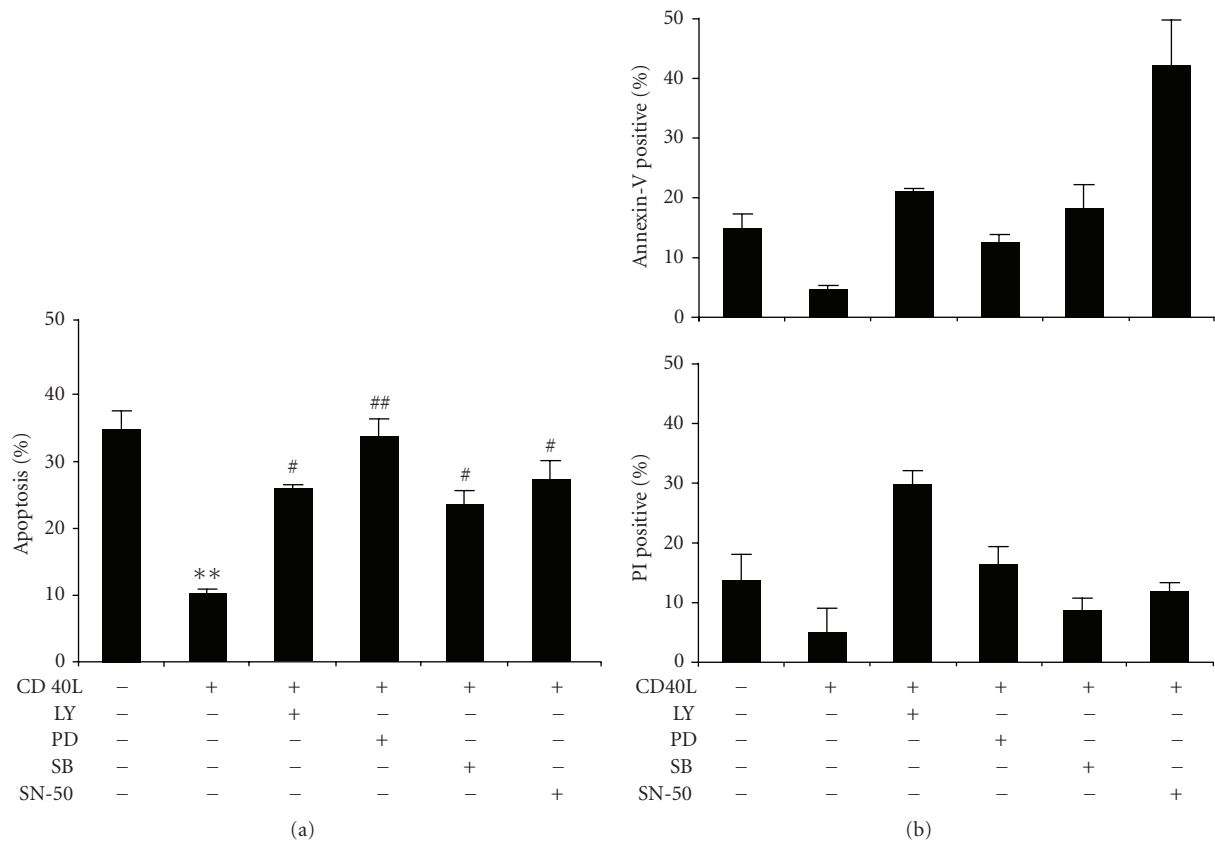


FIGURE 3: Oxaprozin-induced apoptosis involves the inhibition of CD40L-induced Akt and NF-κB phosphorylation. Monocytes were incubated for 48 hours with control medium or 200 ng/mL CD40L plus 1 μg/mL CD40L enhancer in the presence or absence of 50 μM LY294002 (LY), 50 μM PD98059 (PD), 1 μM SB203580 (SB), or 20 μM SN-50. (a) Assessment of apoptosis on cytopreps stained with acridine orange. Data are expressed as percentages of apoptotic cells on total number of cells counted (mean ± SEM, n=3, apoptosis in absence versus in presence of CD40L: **P<.01; apoptosis in presence of CD40L versus CD40L plus PD: ##P<.01; apoptosis in presence of CD40L plus LY or SB or SN-50: #P<.05). (b) Assessment of apoptosis using Annexin V and Propidium Iodide (PI) binding assay. Data are expressed as percentages of positive cells on total number of cells counted (mean ± SEM, n=2). (c) Monocytes were preincubated in the presence or absence of 100 μM oxaprozin for 1 hour and then stimulated with control medium or 200 ng/mL CD40L plus 1 μg/mL CD40L enhancer for 15 minutes. Then, cells were lysed, and western blot analysis was performed. Results represent one of three experiments that yielded similar results.

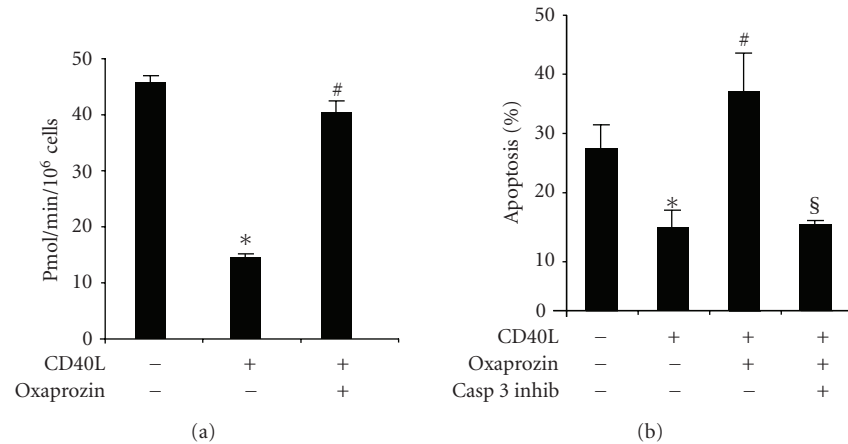


FIGURE 4: Oxaprozin-induced apoptosis of CD40L-treated monocytes involves the activation of caspase 3. (a) Monocytes were cultured for 48 hours with control medium or 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer in the presence or absence of 100 μ M of oxaprozin. Then cells were lysed, and caspase 3 activity was determined by spectrophotometer. Data are expressed as mean \pm SEM, $n=3$. (caspase 3 activity in absence versus in presence of CD40L: * $P<.05$; caspase 3 activity in presence of CD40L versus CD40L plus oxaprozin: # $P<.05$). (b) Monocytes were incubated for 48 hours with control medium or 200 ng/mL CD40L plus 1 μ g/mL CD40L enhancer in the presence or absence 100 μ M of oxaprozin and 50 μ M Ac-DEVD-CHO (caspase 3 inhibitor, CASP 3 INHIB). Data are expressed as mean \pm SEM, $n=3$ (apoptosis in absence versus in presence of CD40L: * $P<.05$; apoptosis in presence of CD40L versus CD40L plus oxaprozin: # $P<.05$; apoptosis in presence of CD40L plus oxaprozin versus CD40L plus oxaprozin and caspase 3 inhibitor: § $P<.05$).

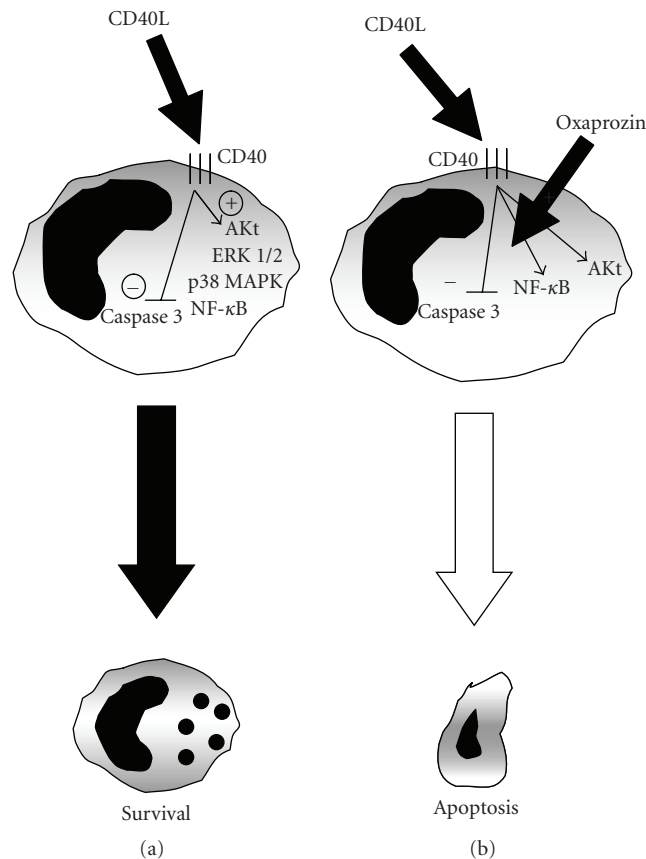


FIGURE 5: (a) CD40L-induced monocyte survival is associated with Akt, ERK 1/2, P38 MAPK and NF- κ B activation, and caspase 3 inhibition. (b) Oxaprozin-induced apoptosis in CD40L-treated monocytes is associated with the inhibition of Akt and NF- κ B and the activation of caspase 3 pathways.

synovial cell COX-2 were, respectively, $2.2 \pm 0.56 \mu\text{M}$ and $36 \pm 28 \mu\text{M}$. These concentrations were similar to IC_{50} previously reported for the other NSAIDs in intact cells [19]. Therefore, although oxaprozin biological activities are commonly explained by the inhibition of COX, the lack of proapoptotic activity of other NSAIDs suggests that oxaprozin-induced increase of apoptosis in CD40L-treated monocytes was not mediated by the inhibition of COX. Then, we focused on COX-independent pathways [11]. Oxaprozin-mediated beneficial effect on monocyte apoptosis is associated with the inhibition of Akt and NF- κB and the activation of caspase 3 pathways. This was suggested by two different findings: (1) CD40L-induced monocyte survival was associated with the activation of PI3K-Akt, p38 MAPK, NF- κB , and ERK 1/2 [34–38], and the activation of all intracellular proteins was required to observe the increased survival; (2) oxaprozin-increased apoptosis of CD40L-stimulated monocytes was associated with the inhibition of Akt and NF- κB . Apoptosis experiments with intracellular protein inhibitors clearly indicate that each intracellular molecule represents a potential target for the pharmacological inhibition of CD40L-induced proinflammatory effects. Although the inhibition of NF- κB was previously reported, the association between oxaprozin treatment and Akt inhibition in CD40L-stimulated monocytes represents a novel finding. Surprisingly, we also showed that oxaprozin treatment was associated with an increase of CD40L-induced ERK 1/2 and p38 MAPK phosphorylation. No significant increase/inhibition in comparison with control medium was observed on ERK 1/2 or p38 MAPK phosphorylation in the presence of oxaprozin alone. Thus, the pharmacological increase of the activation of these kinases represents an unexpected and novel finding, not completely understood. Synthetic anti-inflammatory drugs have been recently shown to increase MAPK activation in other cell types [39, 40]. However, much remains to be clarified in this controversial “activatory” approach [41]. Thus, it could represent an interesting field for future investigations. Finally, the present article showed that oxaprozin treatment was associated with an increase of caspase 3 activation in CD40L-treated human monocytes. This has to be considered as a less specific mechanism of apoptosis induction. In fact, caspase 3 represents the common final pathway of apoptosis, in which converges several pathways [42] and might correspond to upstream oxaprozin-mediated events.

5. Conclusions

Oxaprozin has been shown as an effective drug in the clinical management of adult rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, soft tissue disorders, and postoperative dental pain [43]. In particular, oxaprozin treatment showed better improvement in shoulder function and in the mental health item of the SF-36 quality of life component in comparison with diclofenac [44]. Beneficial effects of oxaprozin might be due to the high rate of accumulation in inflamed synovium in comparison with plasma and synovial fluid levels [33]. On the other hand, oxaprozin might be

also a more active drug than other NSAIDs. Both anti-inflammatory and analgesic properties of oxaprozin are mainly due to the potent inhibition of COX. However, oxaprozin-induced benefits might be also regulated by other COX-independent pathways [11]. This approach could partially explain the superior role of oxaprozin in comparison with other NSAIDs. The present paper shows that oxaprozin induced direct proapoptotic effects in CD40L-treated human primary monocytes independently of COX inhibition. The modulation of apoptotic mediators (such as Akt, NF- κB , and caspase 3) was associated with oxaprozin-mediated proapoptotic effects (Figure 5). Although highly speculative, these data support oxaprozin as a promising pharmacologic agent to suppress monocyte survival and their deleterious activities in chronic inflammatory disorders, such as rheumatoid arthritis.

Acknowledgments

This research was funded by EU FP7, Grant no. 201668, AtheroRemo and supported by a Grant from the Swiss National Science Foundation to Dr. F. Mach (no. 310030-118245). There is no conflict of interest to be declared. Dr. Franco Dallegri received research funding from Helsinn Healthcare SA (Pambio-Noranco, Switzerland) as a part of contribution to the present work.

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