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Tumor necrosis factor-alpha induced caspase-3 activation-related iNOS gene expression in ADP-activated platelets

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Abstract: Platelets are sensitive cells and are easily activated by different stimulants in the circulation system. It is known that tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine and plays a role in inflammation. The role of TNF-α in the apoptotic process in blood platelets is unknown. In order to study the formation of apoptosis in platelets after incubation with TNF-α and/or ADP, several biomarkers were chosen: phosphatidylserine (PS) exposure and P-selectin binding; cGMP, Cyt-c, and Ca^{2+} levels and NOS activation; and gene and protein expression of caspase-3 and iNOS. Platelets were incubated with 100 pg/mL TNF-α and/or 50 mM iNOS specific inhibitor, such as at 1400 W for 1 h at 37 °C in the presence of 5 μM ADP. According to the results, the levels of PS exposure and P-selectin binding were significantly higher in TNF-α + ADP platelets, which decreased with the addition of 1400 W. A significant induction in cGMP, Cyt-c, and Ca^{2+} levels was observed in platelets treated with TNF-α + ADP. On the other hand, the upregulation of the apoptotic gene caspase-3 and iNOS expression levels in TNF-α-treated and ADP-activated platelets was significantly reversed with the addition of 1400 W. These data demonstrate that TNF-α promotes iNOS expression through caspase-3 stimulation in human platelets, and its concomitance leads to the triggering of apoptosis-mediated inflammation upon platelet activation.

Key words: Platelets, apoptosis, TNF-α, iNOS, caspase-3

1. Introduction

Tumor necrosis factor-alpha (TNF-α) is a potent proinflammatory cytokine that plays many roles as a signaling molecule responsible for a diverse range of signaling within cells and as an effector molecule in cellular proliferation, differentiation, or cell death. TNF-α interacts with two distinct cell surface receptors: 55-kDa TNF-R1 and 75-kDa TNF-R2 (Wajant et al., 2003). TNF-R1 is widely expressed in nucleated cell types, and TNF-R2 is expressed mainly in immune and endothelial cells. Platelets are small anucleated cells produced during the blood cell formation in bone marrow, which plays a critical role in thrombus formation under both physiological and pathological blood flow conditions (Deutsch et al., 2006). Platelets stay in the blood circulation for several days and aggregate in response to stimulants such as thrombin, collagen, ADP, and epinephrine (Egger et al., 1998; Yip et al., 2004). Platelets are reported to undergo apoptosis in vitro and in vivo, although they are known to be anucleated cells (Josefsson et al., 2012). There are two major routes by which apoptosis can be induced: the mitochondrial or intrinsic apoptosis pathway, and the death receptor-mediated or extrinsic apoptosis pathway. The intrinsic and extrinsic apoptotic pathways converge at the level of caspase-3 activation. Platelets show many of the signs of the apoptotic mechanism, such as depolarization of the mitochondrial membrane potential, microparticle formation, activation of caspase-3, and exposure of phosphatidylserine (PS) on the platelet surface (White et al., 2010). Nitric oxide (NO) is an important signaling molecule in the body and plays a role in a variety of biological processes. NO is biosynthesized endogenously from L-arginine and molecular oxygen in a reaction catalyzed by various nitric oxide synthase (NOS) enzymes and by the reduction of inorganic nitrate. Three isoenzymes of NOS have been described: type I (neuronal isoform, nNOS), type II (inducible isoform, iNOS), and type III (endothelial isoform, eNOS), displaying about 50% amino acid sequence homology (Gaston et al., 1994). NO is an inhibitor of platelet adhesion, with aggregation produced either by endothelial cells or by the platelets themselves, and it contributes to vessel homeostasis by inhibiting

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vascular smooth muscle contraction and formation of leukocyte–platelet aggregates (Gkaliagkousi et al., 2009). The expressions of both eNOS and iNOS isoforms of NOS have been described in platelets, especially eNOS as a predominant form (Mehta et al., 1995). NO can be produced by platelets, and although its role has been uncertain, it is generally suggested that it plays an important role in modulating platelet function (Gkaliagkousi et al., 2009). Furthermore, a high concentration of NO stimulation from iNOS plays a critical role in proapoptotic response and cell damage, yet its function in various cell types is not clear (Kolb et al., 2000; Bian et al., 2012; Han et al., 2015). The aim of our study was to investigate the effects of TNF-α on platelet apoptosis. The main goal of the study was to detect TNF-α-induced caspase-3 stimulation-related iNOS gene expression in platelets under in vitro conditions.

2. Materials and methods

2.1. Platelet preparation

Fresh ACD-anticoagulated blood was obtained from 12 healthy volunteers (25–40 years of age) with informed consent according to the Ethics Committee (No. 2610201006) of Marmara University, Turkey. Volunteers were not treated with any antiplatelet or antiinflammatory drug. Platelet-rich plasma (PRP) was separately prepared by centrifugation at 150 × *g* for 15 min at room temperature. The PRP was then recentrifuged at 800 × *g* for 15 min to concentrate platelets, and the pellet was resuspended in modified Tyrode's Ca^{2+}/Mg^{2+}-free buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5.6 mM glucose, pH 7.4, and 1 U/mL apyrase) to obtain a final platelet concentration of 2×10^8/mL. The platelet suspension was filtered for the removal of leukocytes using a special filter (Imugard, Terumo). Platelet counting was performed with a hemocytometer, and platelet viability and leukocyte contamination were assessed by performing cytologic studies with trypan blue and May-Grünwald-Giemsa stains (95%–99% vital platelets). Platelets were suspended with HEPES buffer and incubated with a 50 mM, 1400 W iNOS inhibitor (Cayman, USA) or with 100 pg/mL TNF-α (Invitrogen, CA) for 1 h at 37 °C in the presence of 5 μM ADP. Adenosine diphosphate (ADP) was used for the stimulation of platelets.

2.2. Platelet activation and phosphatidylserine exposure assay

Platelet-surface exposure of PS was determined with the Annexin V-PI Apoptosis Detection Kit (BD, USA) using flow cytometry according to the manufacturer's instructions. Platelet suspensions of 100 μL aliquots each were placed in siliconized glass cuvettes for PS exposure experiments and were combined with fluorescein isothiocyanate (FITC)-conjugated annexin V and phycoerythrin (PE)-conjugated propidium iodide. They were then incubated for 15 min at RT. Samples were diluted 5-fold with binding buffer for immediate analysis by flow cytometry.

For the determination of platelet activation, P-selectin expression was monitored as described previously (Sener et al., 2012). Platelets were fixed with 2% paraformaldehyde and incubated for 15 min at RT with 1 μg/mL FITC-labeled anti-CD62P and were examined by flow cytometry. For the flow cytometry analyses, all the samples prepared as described above were analyzed with the Becton-Dickinson FACScan. The flow cytometer was equipped with a 488 nm argon ion laser. The platelet population was identified by forward scatter for cell size and by side scatter for cell granularity. The alignment of the instrument was checked daily with calibration beads. An electronic bitmap was placed around the platelet population and CD41a-FITC was used to form a gate. The results were evaluated as the percentage of antibody-positive platelets. FITC-conjugated immunoglobulin G (IgG) was used to determine the nonspecific and background fluorescence. Fifty thousand platelets were counted in each tube.

2.3. Measurement of Ca^{+2} and Cyt-c

Intracellular calcium mobilization of the platelets was measured with a Fluo-8 No-Wash Calcium Assay Kit (AAT Bioquest, USA). Fluo-8 solution was added to the platelet suspensions and the mixture was incubated for 30 min at 37 °C. Ca^{2+} levels were determined at Ex/Em = 490/525 nm with a fluorescence microplate reader (Glomax, Promega, USA). Cytochrome c (Cyt-c) was shown to redistribute from mitochondria to the cytosol during apoptosis in cells. The measurement of Cyt-c release in platelets was performed with the human Cyt-c ELISA Kit (eBioscience, Germany) as described previously (Dasgupta et al., 2010; Cevik et al., 2013). Platelets were resuspended with lysis buffer and then centrifuged at 18,000 × *g* for 10 min. Platelet supernatant included cytosolic proteins and was analyzed by manufacturer protocols with an ELISA reader (BioTek, USA). Total protein concentration in the samples was quantified using a Bradford protein assay kit.

2.4. Measurement of cGMP and total NOS activity

Cyclic guanosine monophosphate (cGMP) content was determined by measuring the generation of cGMP in platelets with a standard cGMP ELISA kit (eBioscience, Germany) according to the protocols recommended by the manufacturer. Samples were run in triplicate, relative to the cGMP standard curve by use of a microplate reader (Epoch, BioTek, USA). Results are expressed as picomoles of cGMP per milligram of total protein. Total NOS activity was measured with a kit (Oxford Biomedical Research, NB78, USA) according to the manufacturer's instructions. NOS activity was determined with recombinant nitrate reductase, converting nitrate to nitrite by Griess reagent.

By using sodium nitrite as the standard curve, the amount of nitrate conversion to nitrite was measured at 540 nm.

2.5. Western blot analysis for caspase-3 and iNOS

Platelet lysates were suspended in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% Nonidet P40, 0.25% SDS leupeptin, and aprotinin) and centrifuged for 15 min at 3000 × *g* at 4 °C. The supernatant was collected and the protein concentration was determined with the Bradford protein assay kit. After adjustment for protein concentration, cell lysates (30 µg protein) were boiled in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, containing 0.05% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromphenol blue) and resolved on 12% SDS-PAGE. They were then transferred to nitrocellulose membrane, as described previously (Cevik et al., 2013). The membrane was blocked with 5% nonfat skim milk powder in Tris-buffered saline (TBS) containing 0.1% Tween-20. The membrane was washed twice in TBST (5 min each) and incubated overnight with the primary antibody (1:500 monoclonal human anticaspase-3, anti-iNOS, b-actin). The membrane was washed three times (5 min each) in TBST and then incubated with HRP-conjugated secondary antibody for 2 h. After being washed three times (5 min each), the blot was developed with a Western Blotting Enhanced Chemiluminescence Luminol Reagents Kit and exposed to a film to visualize the protein bands that were scanned with an imaging system (Kodak, DC290, USA). A representative blot for each protein of interest is shown, and β-actin was used as a protein-loading control. Band intensities were quantified by determining the area under the curve for each band (ImageJ software 1.43m, National Institutes of Health, Bethesda, MD, USA). Band intensities were normalized to the intensity of the corresponding control, and the mean and SD values are shown.

2.6. Quantitative reverse transcriptase real-time PCR

2.6.1. RNA extraction and reverse transcription

Total RNA was isolated using NucleoSpin RNAII in compliance with the manufacturer's instructions. Total RNA concentrations were determined using the Take3 Multi-Volume Plate (BioTek, USA) according to the manufacturer's instructions. A260/A280 ratios were >1.9, and RNA was stored at –70 °C.

cDNA synthesis was performed using a First-Strand cDNA Synthesis Kit according to the manufacturer's instructions. Reverse transcription of 0.1 µg of total RNA was carried out in a total volume of 20 µL reaction mixture, consisting of 10 mM dNTP mix, a reaction buffer, 1 µL random hexamer, 20 U/µL RNase inhibitor, and 20 U/µL MuLV reverse transcriptase. Samples were incubated at 25 °C for 5 min, at 37 °C for 60 min, at 70 °C for 5 min, and finally at 4 °C for 5 min in a GenAmp PCR System 9700 thermal cycler (PE Applied Biosystems, USA).

2.6.2. PCR conditions

Real-time PCR was performed using SYBR Green SuperMix with approximately 100 ng of cDNA and primers in a total volume of 20 µL reaction mix, as described previously (Cevik et al., 2013). PCR was performed at 94 °C for 5 min, and then 50 cycles for caspase-3 were run at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. For iNOS, 50 cycles were run at 94 °C for 30 s, 60 °C for 30 s, and 74 °C for 35 s. For GAPDH, 40 cycles were run at 94 °C for 40 s, 60 °C for 30 s, and 68 °C for 40 s on the iQ™5 Optical System (Bio-Rad). At the end of the amplification cycles, melting temperature analysis was carried out with a slow increase in temperature transition rate of 0.1 °C/s up to 95 °C. The primers used were: iNOS, 5'CAGCGGGATGACTTTCCAA3' and 5'TGCAGGTCCAAATCTTGCCT3'; caspase-3, 5'CAGTGGAGGCCGACTTCTTG3' and 5'ATCCAGTCGCTTTGTGCCA3'; GAPDH, 5'TGCACCACCAACTGCTTAGC3' and 5'GGCATGGACTGTGGTCATGAG3'. Amplification, data acquisition, and analysis were performed using Qbase (Bio-Rad, USA). Coupled to the instrument, the software determines the threshold cycle that represents the number of cycles in which fluorescence intensity is significantly above the background fluorescence. The amounts of caspase-3 relative to the amount of GAPDH in the same sample were analyzed with the relative quantification approach. The results are expressed as normalized ratios.

2.7. Statistics

Statistical analysis used multivariate analysis of variance. Data are presented as the mean ± SD from N independent experiments. A two-tailed, paired Student's t-test was used to compare the test and the control groups. The threshold for statistical significance was set to $P < 0.05$.

3. Results

3.1. Platelet activation with TNF-α

Platelet activation was determined quantitatively by measuring the production of soluble P-selectin. The changes of the platelet activation markers are shown in Figure 1A (platelet samples stained with CD62p). The percentage of CD62p exposure in the ADP group (24.78 ± 4.21%) was significantly different from that of the control (16.33 ± 2.25%) ($P < 0.05$). CD62p levels of the TNF-α + ADP group (44.66 ± 6.03%) were significantly increased compared to the ADP ($P < 0.01$). CD62p levels of the TNF-α + ADP + 1400 W group (32.45 ± 6.05%) decreased compared to the TNF-α + ADP group, but not significantly (Figure 1B).

Platelet PS expressions, i.e. early apoptotic markers, are shown in Figure 1C. Using the annexin-V-FITC antibody against the platelets, PS expression was measured in groups by histograms (Figure 1C). PS expression levels in the ADP group (7.95 ± 2.21%) were significantly higher

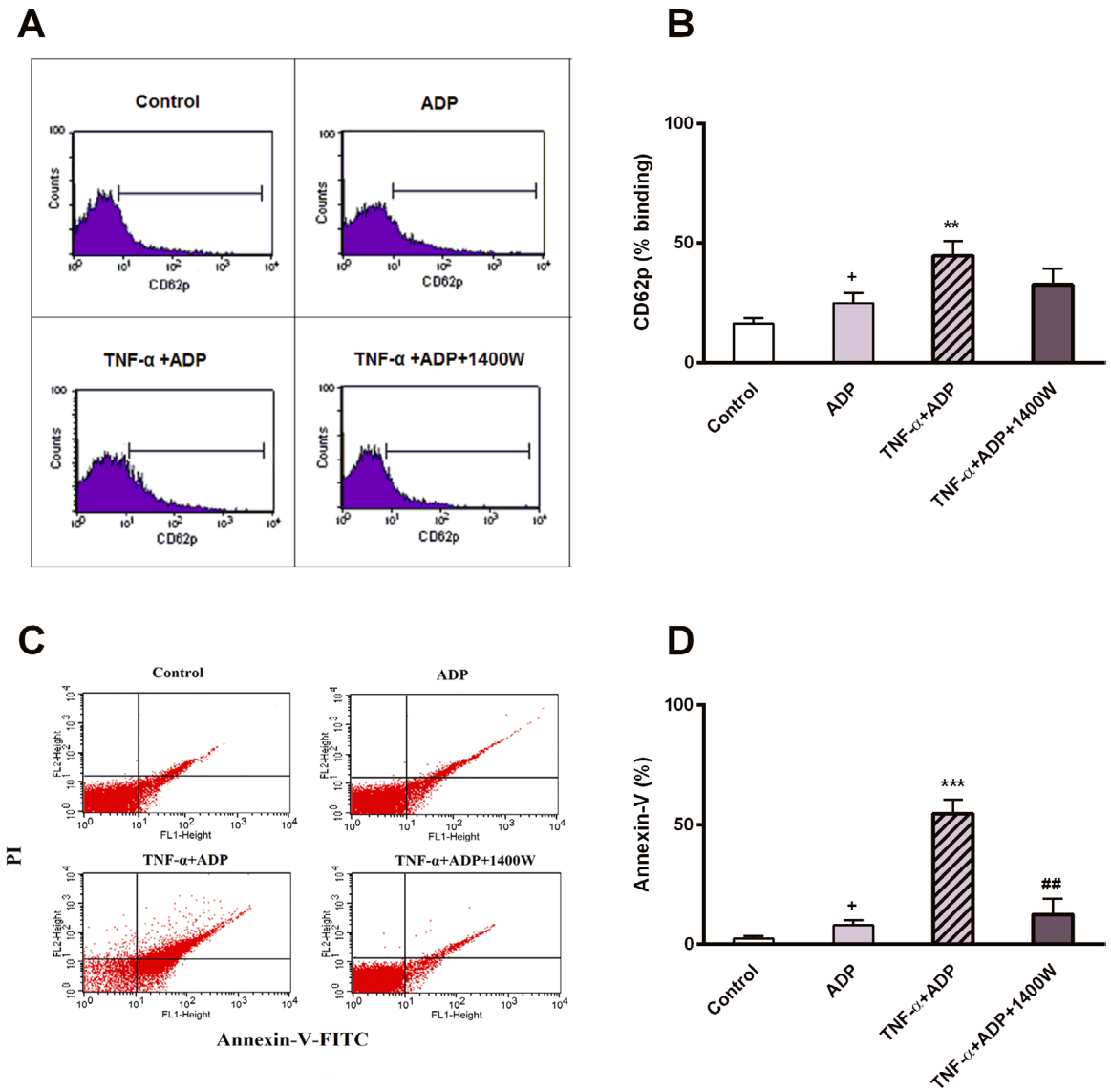

Figure 1. A) Histogram samples of control, ADP, ADP + TNF-α, and ADP + TNF-α + 1400 W groups for P-selectin measurement by flow cytometry. B) Mean values of P-selectin in platelets (+: $P < 0.05$ versus control group, **: $P < 0.01$ versus ADP group). C) PS exposure of platelets was determined using annexin-V-FITC in samples of control, ADP, ADP + TNF-α, and ADP + TNF-α + 1400 W groups. D) Mean values of annexin-V in platelets (+: $P < 0.05$ versus control group, ***: $P < 0.01$ versus ADP group, ##: $P < 0.01$ versus TNF-α + ADP group).

than in the control group (2.33 ± 1.25%) ($P < 0.05$). In the TNF-α + ADP group (54.52 ± 6.01%), PS expression levels were significantly higher than in the ADP group ($P < 0.001$), and in the TNF-α + ADP + 1400 W group (12.45 ± 6.65%), PS was significantly lower than in the TNF-α + ADP group ($P < 0.01$) (Figure 1D).

3.2. The effects of TNF-α on the Ca^{2+} oscillation and Cyt-c release in platelets

The contribution of the internal Ca^{2+} was monitored in incubated platelets (Figure 2A). ADP-activated platelets showed higher Ca^{2+} levels than the control platelets ($P < 0.001$). The internal Ca^{2+} levels in the TNF-α + ADP group were increased compared to the ADP group ($P < 0.001$). On the other hand, the addition of 1400 W to TNF-α + ADP-induced platelets caused significant reductions in Ca^{2+} levels compared to the TNF-α + ADP group ($P < 0.001$).

Cyt-c levels of platelets are shown in Figure 2B. After activation with ADP, there was a significant increase in the Cyt-c levels as compared to the control platelets ($P < 0.05$). The addition of TNF-α in ADP-induced platelets caused significant inductions in Cyt-c levels ($P < 0.01$). In

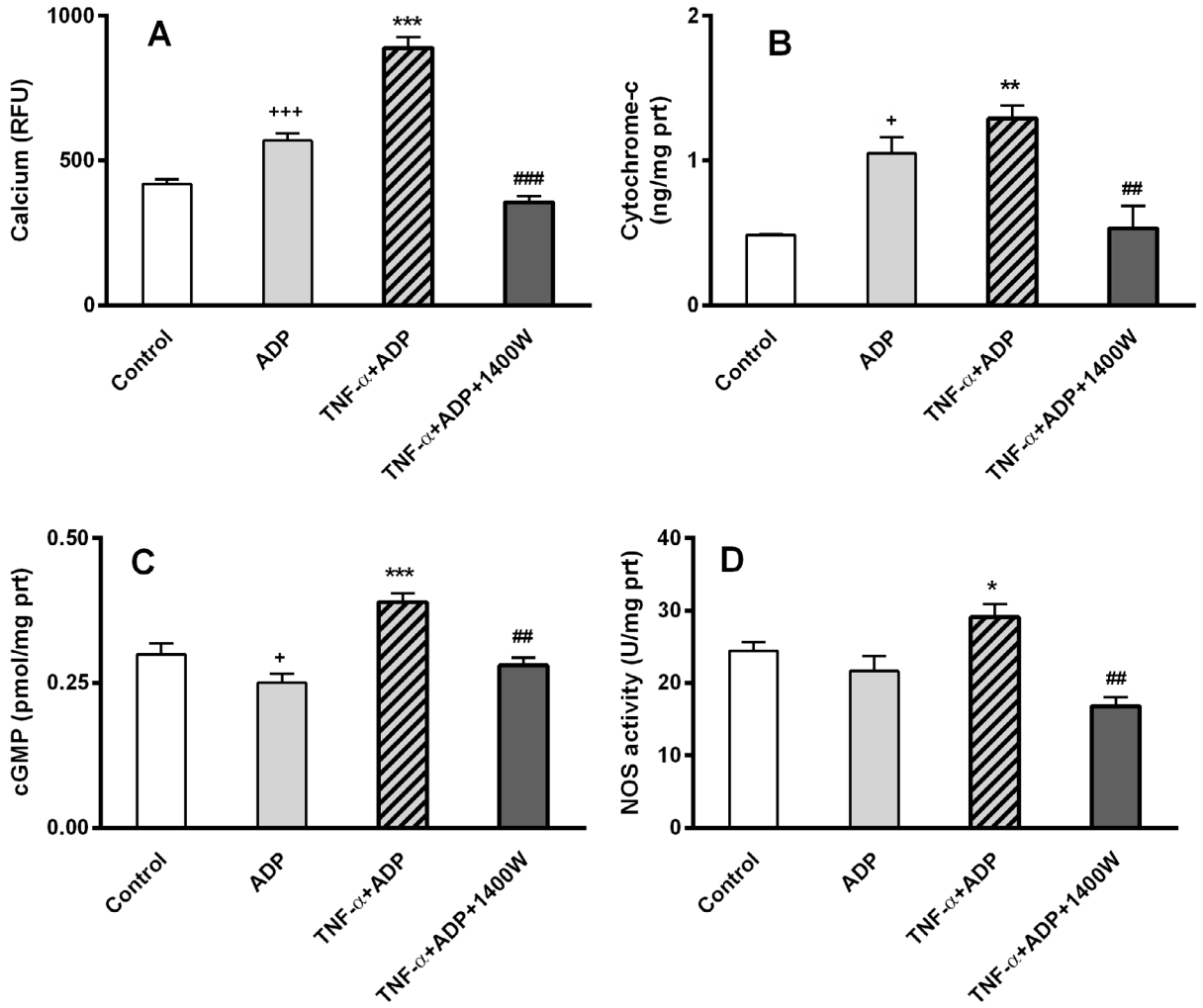

Figure 2. A) Platelet Ca^{+2} levels (+++: P < 0.001 versus control group, ***: P < 0.001 versus ADP group, ##: P < 0.001 versus TNF-α + ADP group). B) Platelet Cyt-c levels (+: P < 0.05 versus control group, **: P < 0.01 versus ADP group, ##: P < 0.01 versus TNF-α + ADP group). C) Platelet cGMP levels (+: P < 0.05 versus control group, ***: P < 0.001 versus ADP group, ##: P < 0.01 versus TNF-α + ADP group). D) Platelet NOS activity levels (*: P < 0.05 versus ADP group, ##: P < 0.01 versus TNF-α + ADP group).

addition, Cyt-c levels increased after the addition of 1400 W to TNF-α + ADP-induced platelets compared to the TNF-α + ADP group (P < 0.01).

3.3. The effects of TNF-α on the nitric oxide metabolism in platelets

The results of nitric oxide metabolism markers of CGMP and total NOS activity are shown in Figures 2C and 2D. To find out which signaling pathway might mediate the stimulatory effects of TNF-α, platelets were pretreated for 10 min with 50 mM and 1400 W, followed by 1 h of incubation with TNF-α + ADP. cGMP levels in the ADP group were decreased compared to the control group. TNF-α increased cGMP production in ADP-activated platelets (P < 0.001). cGMP levels in platelets were decreased for the 1400 W + TNF-α + ADP group compared to the TNF-α + ADP group (P < 0.01). In contrast, the ADP-activated platelets were not different in terms of total NOS activity compared to the control group. However, there was a reduction trend in NOS activity. A significant increase in NOS activity was observed for the TNF-α + ADP group compared to the ADP group (P < 0.05), and a significant decrease was observed for the ADP + TNF-α + 1400 W group compared to the TNF-α + ADP group (P < 0.001).

3.4. Effects of TNF-α on the caspase-3 and iNOS gene and protein expression in platelets

As shown in Figure 3A, caspase-3 gene expression levels were slightly higher in ADP-activated platelets compared to the control group (P < 0.05). Results showed that the mRNA of caspase-3 in the TNF-α + ADP group was

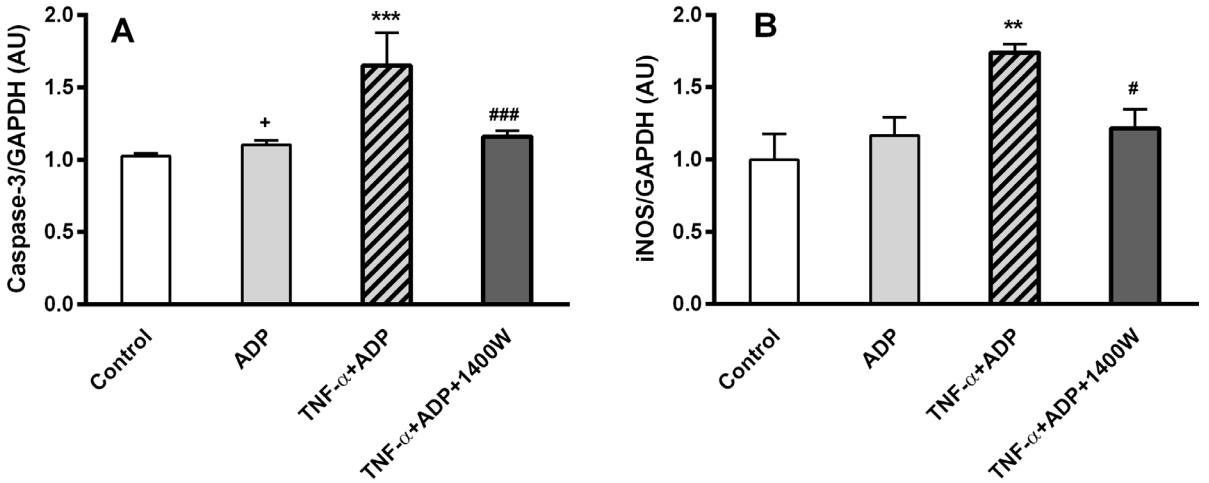

Figure 3. A) Caspase-3 gene expression in platelets determined by qRT-PCR (+: $P < 0.05$ versus control group, ***: $P < 0.001$ versus ADP group, ###: $P < 0.001$ versus TNF-α + ADP group). B) iNOS gene expression in platelets determined by qRT-PCR (**: $P < 0.01$ versus ADP group, #: $P < 0.05$ versus TNF-α + ADP group).

significantly higher than in the ADP group ($P < 0.001$). Treatment with 1400 W showed the highest reduction in caspase-3 gene expression. These observations were consistent with the results derived from real-time RT-PCR ($P < 0.001$). iNOS gene expression levels of the platelets are shown in Figure 3B. iNOS gene expression in ADP-activated platelets was slightly higher than in the control platelets, although the results were not statistically significant. After presenting TNF-α in ADP-activated platelets, iNOS gene expression levels were significantly higher ($P < 0.01$). iNOS gene expression levels decreased with the addition of 1400 W in the TNF-α + ADP group ($P < 0.05$).

Caspase-3 and iNOS protein expressions detected with western blotting are shown in Figure 4A. β-Actin was used as an internal control. The data are presented as mean ± SD, performed in triplicate. Caspase-3 is expressed as an inactive 32-kDa precursor, of which the p20 and p11 subunits are proteolytically generated during the onset of apoptosis. Western blot analysis of the p11 subunit of the cleaved caspase-3 showed an 11-kDa band in ADP-stimulated platelets. The p11 subunit of the cleaved caspase-3 bands was greater in the TNF-α + ADP group compared to the ADP group. Caspase-3 protein expression of ADP-activated platelets was significantly elevated compared to the control group, indicating activation-induced apoptosis ($P < 0.05$; Figure 4B). After TNF-α, there was a significant difference in the protein expression of caspase-3 of the platelets compared to the ADP group ($P < 0.01$). On the other hand, TNF-α induced an increase in caspase-3 and it was significantly depressed upon the addition of 1400 W in the TNF-α + ADP group ($P < 0.001$).

Western blot analysis of iNOS revealed clear 130-kDa bands in the TNF-α + ADP-incubated platelets and only faint bands for 1400 W incubation in the TNF-α + ADP group (Figure 4A). There was no change in iNOS protein expression levels in ADP-activated platelets compared to the control group (Figure 4C). The level of iNOS protein expression in TNF-α + ADP-induced platelets was significantly higher than in ADP-activated platelets ($P < 0.001$). In contrast, iNOS protein expression levels were relatively decreased when 1400 W was added to TNF-α + ADP incubated platelets ($P < 0.001$).

4. Discussion

Platelets, known as anucleated cells, are able to undergo programmed cell death in response to diverse stimulants. Platelets arise from the intrinsic apoptotic pathway characterized by depolarization of mitochondrial inner transmembrane potential and express several components of the apoptotic machinery, such as activation of caspase-3, caspase-8, and caspase-9; PS externalization and shedding of microparticles; and proapoptotic protein expression of Bax and Bcl-2 (Leytin et al., 2009; Vogler et al., 2011). Platelet apoptosis and activation have been determined through well-known markers of these platelet responses by flow cytometry as PS externalization and CD62p exposure, respectively (Gyulkhandanyan et al., 2012). P-selectin is stored in α-granules of platelets, rapidly expressed on the platelet cell surface during activation. CD62p acts as a ligand to generate proinflammatory and procoagulatory platelet–leukocyte aggregates (Michelson et al., 1996). Our findings demonstrated that CD62p and PS expression levels in platelets increased in the presence of TNF-α. Induction of platelet PS exposure and microparticle release is generally linked to a Ca-dependent mechanism. Intercellular calcium signaling is a common form of cell–cell interaction that enables tissue coordination of cell

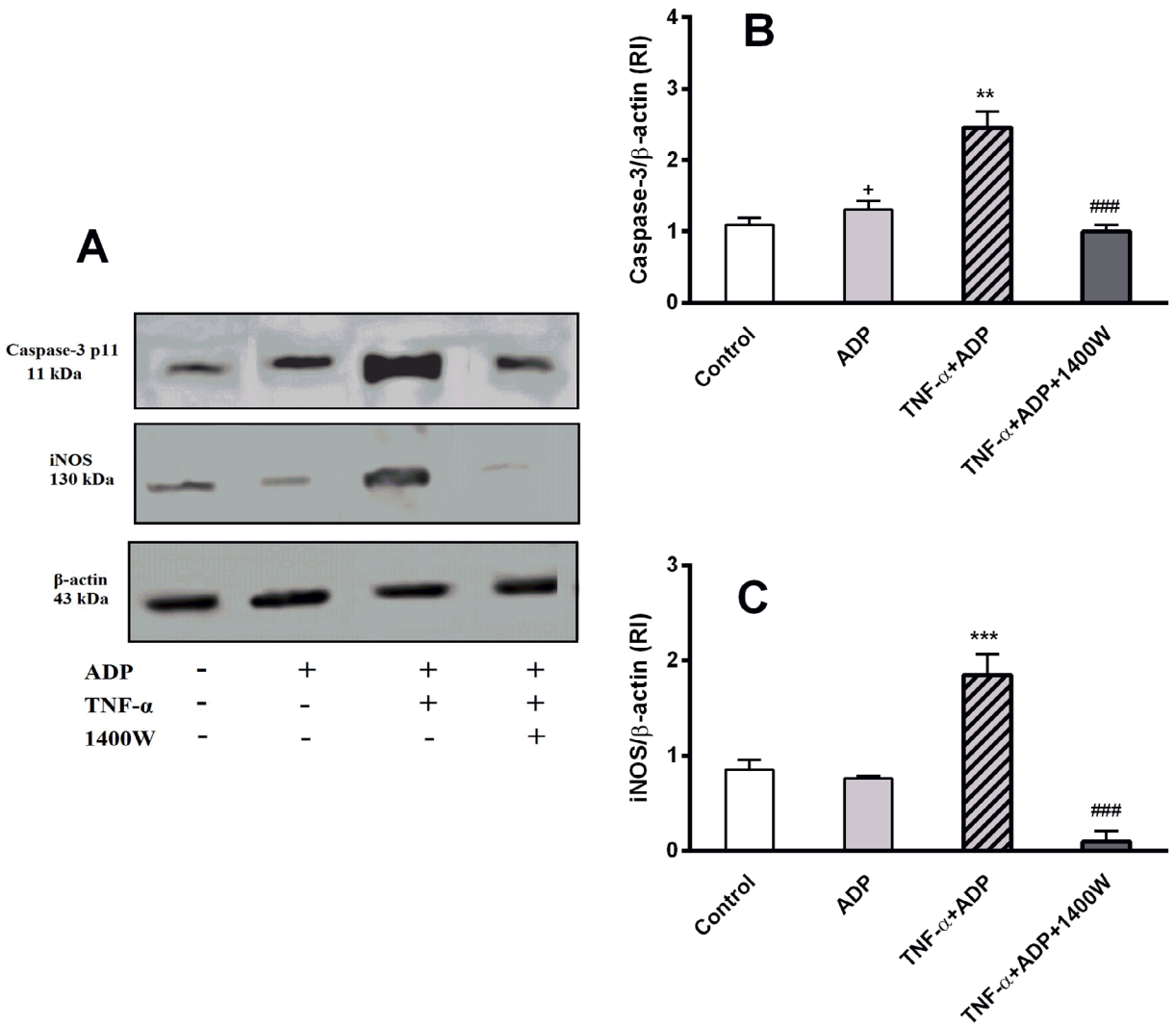

Figure 4. A) Activation of caspase-3 and iNOS protein expression by TNF-α in ADP-stimulated platelets determined by western blotting. B) Caspase-3/β-actin ratio in the platelets (+: $P < 0.05$ versus control group, **: $P < 0.01$ versus ADP group, ###: $P < 0.001$ versus TNF-α + ADP group). C) iNOS/β-actin ratio in the platelets (***: $P < 0.001$ versus ADP group, ###: $P < 0.001$ versus TNF-α + ADP group).

proliferation, differentiation, and metabolism (Resende et al., 2013). In the presence of proinflammatory cytokines, such as interleukin-1, IFN-γ, and TNF-α, inducible Ca^{2+}-independent NOS is expressed in vascular smooth muscle cells, leading to persistent generation of large quantities of NO (Yan et al., 1996). It is known that TNF-α can induce Ca^{2+} oscillation, which is set into action by reducing Ca^{2+} signaling pathways (Yarilina et al., 2011). Previous studies have demonstrated that ADP-activated platelets oscillate Ca^{2+}, and the efficiency by which Ca^{2+} signals are spread within the platelet aggregates plays an important role in thrombus growth (Nesbitt et al., 2003). Although Ca^{2+} is known as a key promoter of cell survival, this ion can also stimulate the apoptotic machinery in response to some pathological situations (Hajnoczky et al., 2003). Our findings demonstrated that TNF-α triggers Ca^{2+} oscillation in ADP-activated platelets. Blood platelets do not have nuclei and thus do not have the ability to regulate protein expression transcriptionally; however, platelets include high levels of mRNA when differentiation from megakaryocytes occurs. Because platelets have a greater capacity for protein synthesis, they generate NO when activated by agonists such as adenosine diphosphate, collagen, thrombin, and von Willebrand factor, whereas NO release is lacking in unstimulated platelets (Freedman et al., 1999; Böhmer et al., 2015). We have recently shown that ADP can induce oxidative stress in platelets and activates NOS, which plays an important role in NO synthesis during platelet activation and in stimulation and apoptosis (Sener et al., 2013). Previous studies have shown

that low levels of iNOS were detected in platelets, yet its functional state remained unknown (Wallerath et al., 1997; Marjanovic et al., 2008; Smolenski, 2012). Indeed, we have detected iNOS mRNA expression in human platelets by qRT-PCR. NO production in platelets occurs with TNF-α incubation after ADP addition and is followed with changes in iNOS protein expression levels, denoting a signaling-mediated functional activation mechanism of iNOS. Considerably, iNOS inhibitors reduce TNF-α + ADP-induced cGMP levels in platelets, showing that iNOS activation is critical in stimulating the presence of TNF-α. There are several studies suggesting that TNF-α modulates iNOS expression in a variety of cell types (Heba et al., 2001; Nandi et al., 2010). Here we showed that NO synthesis during platelet activation is also mediated by the constitutively expressed iNOS protein in platelets. On the other hand, it is known that iNOS could produce excessive amounts of NO, whereas nNOS and eNOS could contribute to the production of low levels of NO (Mehta et al., 1995). The functional roles of NOS are different in platelets; for instance, eNOS precludes interactions in the platelet vessel wall and inhibits platelet aggregation, whereas high levels of NO produced from iNOS can trigger oxidative modifications and apoptosis (Osbom et al., 2002; Marjanovic et al., 2008; Sener et al., 2012). It has been reported that NO can regulate P-selectin expression (Keh et al., 1996). In addition, some NOS inhibitors can elevate platelet activation (Armstead et al., 1997). This may be clinically important, as some researchers suggest that levels of NO metabolites and P-selectin expression are linked in platelets (Minamino et al., 1998). In our study, it is confirmed that NOS activation was enhanced with TNF-α incubation in platelets at the same time as expression of iNOS protein increased during platelet activation with TNF-α. Inhibition of the iNOS activity with 1400 W also blocked the decrease in intracellular cGMP, Ca^{2+}, Cyt-c, and NOS levels in this study.

We investigated whether inflammation-induced activation of the iNOS pathway is associated with increased caspase activity and apoptosis. Recent studies demonstrated that there is a synergistic signaling relationship between iNOS and caspase-3 when incubated with some stimulators in a variety of cell types (Andersson et al., 2006; Kiang et al., 2007; Lukes et al., 2008). Caspase-3 is a member of the cysteine-aspartic acid protease family that mediates apoptotic cell death. Regarded as an apoptotic marker, caspase-3 activation is an essential component of many downstream caspases involved in the apoptotic cascade as a determinant in platelet thrombopoiesis (Çevik et al., 2012). Intrinsic programmed cell death-characterized proteins contain Cyt-c, which mediates the activation of caspase-9 and triggers the stimulation of a cytosolic caspase complex, including caspase-3 activation, which promotes cellular apoptosis (Li et al., 1997). Cyt-c is released from the mitochondria into the cytosol during programmed cell death and, additionally, platelets express caspase-3, caspase-9, Apaf-1, and members of the Bcl-2 family (Bertino et al., 2003). We report that TNF-α incubation for 1 h with ADP in human platelets induced caspase-3 protein and gene expression, depending on the rise of both Cyt-c and Ca^{2+} levels. Processing/activation of platelet caspase-3 can be induced by several characterized platelet agonists (Josefsson et al., 2012). Our results are in agreement with previous studies reporting activation of caspase-3 gene expression by ADP (Keh et al., 1996). A number of studies have established that large agonist concentrations provoke early and late caspase-3 activation in apoptosis. Furthermore, agonists at physiological concentrations induce rapid caspase-3 activation independently of the apoptotic pathway that contains Cyt-c, caspase-9, and PS exposure (Shcherbina et al., 1999; Rosado et al., 2003; Böing et al., 2008). On the other hand, caspases are activated in TNF-α-induced cell death, especially activation of caspase-3, known as an executioner. TNF-α can initiate the release of cytosolic Cyt-c, which leads to the activation of caspase-9, evidence that the cell death is mitochondria-dependent (Zhao et al., 2001; Utaisincharoen et al., 2009; Alvarez et al., 2011). Recent studies have suggested that TNF activates platelet caspases via TNF-R1 and demonstrated the correlation between the activation of platelets and thrombocytopenia (Piguet et al., 2002; Yang et al., 2010). As a result, TNF-α significantly induced the cytosolic release of Cyt-c, which contributes to gene and protein expression of caspase-3, meaning that cell death is associated with the mitochondria. In addition, iNOS regulates caspase-3 activation due to high NO production by iNOS in human platelets. Our study also shows that, depending on the triggering stimulus, a high level of platelet activation is associated with platelet apoptosis, and the apoptotic and activation responses may be linked to inflammation and NO synthesis.

Acknowledgments

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