NOX 5 is expressed in platelets from patients with chronic granulomatous disease

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Dear Sirs,

X-linked chronic granulomatous disease (X-CGD) is associated with life-threatening infectious disease, which is related to impaired innate immune system (1). Thus, X-CGD is a very rare disease, which is associated with hereditary deficiency of Nox2, the enzyme involved in the cellular formation of reactive oxidant species (ROS) and eventually bacteria killing (2). Recent studies demonstrated that Nox2 is present not only in leucocytes but also in platelets, where it is implicated in platelet activation via formation of 8-iso-PGF2α (3). In X-CGD patients Nox2 in down-regulated not only in leucocytes but also in platelets but ROS formation is not fully suppressed suggesting the existence of other ROS platelet source (4). However, the enzymatic pathway responsible for such residual ROS formation has not been clarified (5). In addition to Nox2, Nox family encompasses other isoforms such as Nox1, Nox3, Nox4 and Nox5, which contribute to ROS formation in different cell lines (6). There is still uncertainty as to whether platelets express other Nox isoforms (7, 8) and their role on ROS formation.

We studied three healthy subjects (HS; 3 males, age 46 ± 3.08) and three patients (3 males, age 28 ± 8.7) with X-CGD, which was diagnosed as previously described (1). The study was conducted in accordance with declaration of Helsinki. Blood samples from HS and X-CGD patients were taken between 8:00 and 9:00 AM, and collected in tubes with 3.8% sodium citrate (ratio 9:1). To obtain platelet-rich plasma (PRP), samples were centrifuged for 15 minutes (min) at 180g and to prevent leukocyte contamination; only the top 75% of the PRP was collected.

Platelet pellets were obtained by PRP centrifugation (10 min, 300g) after the addition of acid/citrate/dextrose (1:10 vol/vol) to avoid cell activation during processing. Platelet pellets were suspended in Tyrode buffer in the presence of 0.1% albumin, pH 7.35 (2×10^8 platelets/ml, unless otherwise specified.

To evaluate the Nox5 isoform, 50 μg/ml of total protein was analysed by western blot analysis. Western blot analysis was performed with polyclonal anti-Nox5 incubated overnight at 4°C. After incubation, the pure nitrocellulose membranes were washed and incubated with goat anti-rabbit IgG-HRP for 2 hours. Immune complexes were detected by enhanced chemiluminescence. The developed spots were calculated by densitometric analysis on a NIH Image 1.62f analyzer, and the values were expressed as arbitrary units.

The evaluation of Nox5 expression in platelets was also performed by PCR analysis. Briefly, total RNA was extracted (Total RNA Purification Kit, Norgen Bioteck Corp, Thorold, ON, Canada) and reverse-transcribed into complementary DNA (cDNA) using Tetro cDNA Synthesis kit (Bioline, Reagents Ltd, London, UK) (9, 10). Transcript levels were assessed by PCR according to RBC Taq DNA polymerase (RBC Bioscience, New Taipei City, Taiwan) protocol and gene product visualized on 2% agarose gel.

Platelet oxidative stress was measured by ROS production and 8-Iso-PGF2α formation as previously described (11).

In particular, PRP was stimulated for 10 min at 37°C with or without 0.5 mM Arachidonic Acid (AA) in the presence or less of KN-93 (10 μM), an inhibitor of Nox5, or a control peptide (CP) (50 μM); the supernatant was stored at -80°C until use.

Data are reported as mean ± SD. The comparison between variables in the in study was made by the Student t-test for paired and unpaired data. The data were also confirmed by nonparametric test. Bivariate analysis was performed with a spearman correlation test. Significance was accepted at $p<0.05$.

While we were unable to detect Nox1 and Nox4, (data not shown), platelets from HS and X-CGD did express Nox5; western blot analysis of platelets from X-CGD patients showed Nox5 expression with a quantity comparable to that of normal platelets (►Figure 1A). This finding was confirmed by PCR analysis showing that Nox5 is expressed not only in HS and but also in X-CGD (►Figure 1B). We also analysed if other blood cells did express Nox5 but we were unable to find it in leucocytes and monocytes (data not shown).

Upon stimulation platelets from HS produced ROS and 8-iso-PGF2α formation, which were significantly inhibited if incubated with Nox5 inhibitor (-31%, 35.3 ± 5.5 S.I. vs 24.3 ± 4.0 S.I for ROS production and –20%, 170.0 ± 5.1 pmol/l vs 136.0 ± 13.5 pmol/l for 8-iso-PGF2α formation). Platelets from X-CGD showed lower formation of ROS and 8-iso-PGF2α formation compared to control (18.3 ± 2.5 vs 35.3 ± 5.5 S.I and 83.3 ± 7.6 vs 170.0 ± 5.1 pmol/l, respectively, $p<0.001$); both were significantly inhibited in platelets incubated with Nox5 inhibitor (Figure 1C, D). Together this finding indicated that the residual ROS formation in platelets from X-CGD is dependent upon Nox5 acti-

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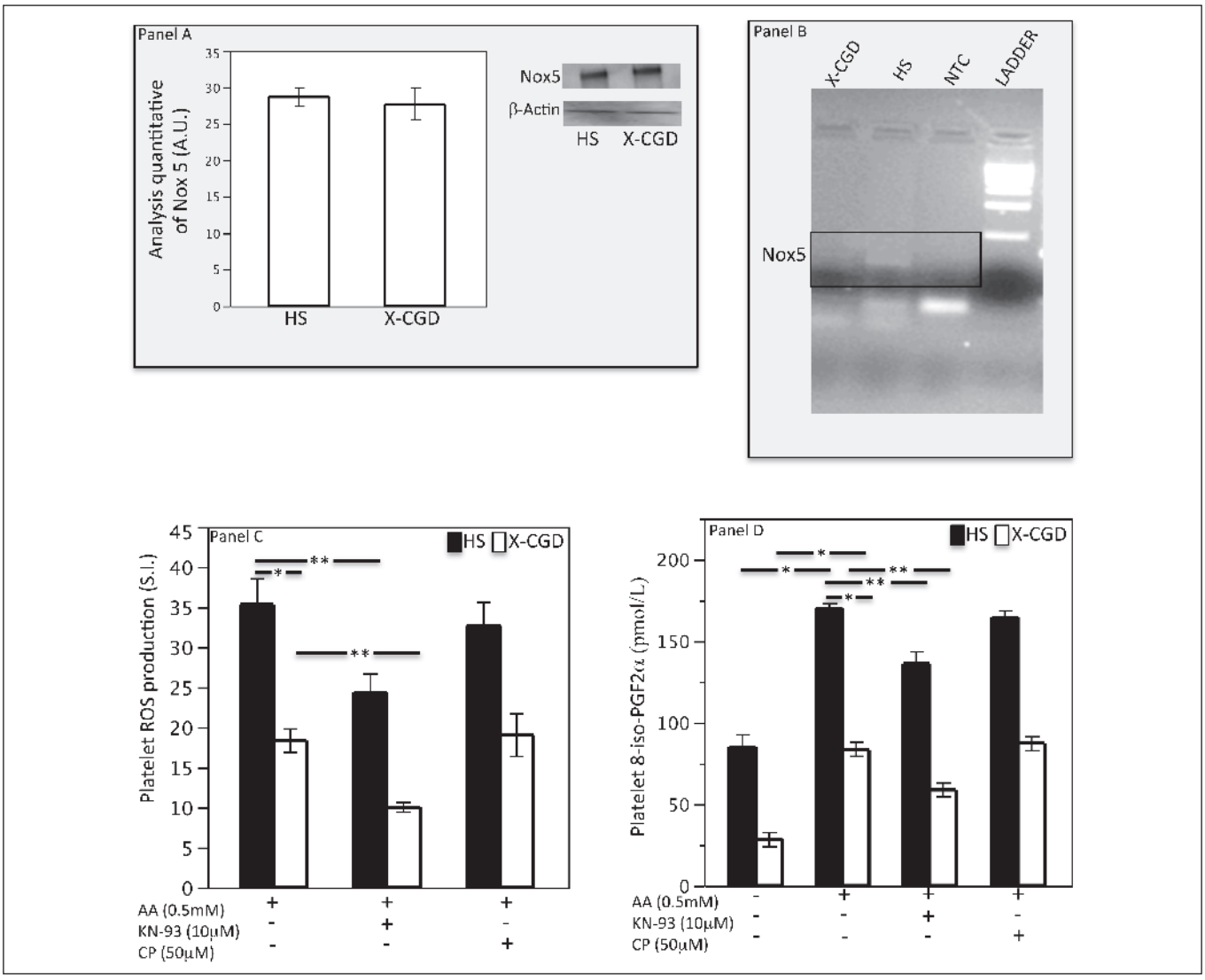

Figure 1: Expression and function of NOX5. A) Quantitative analysis and a representative western blot of Nox5, in platelets of Healthy Subjects (n=3) and X-CGD patients (n=3). B) A representative RT-PCR analysis of Nox5 in platelet of Healthy Subjects and X-CGD patients. C) Platelet ROS production in samples treated with or without Arachidonic Acid (AA 0.5mM) in presence or less of Nox5 specific inhibitor (KN-93 10 μM) or Control Peptide (CP 50 μM) in Healthy Subjects (n=3) and X-CGD patients (n=3). (*p<0.001, **p<0.05). D) 8-Iso-PGF2 formation in supernatant of AA-stimulated platelets incubated with Nox5 specific inhibitor (KN-93 10 μM) or Control Peptide (CP 50 μM) in Healthy Subjects (n=3) and X-CGD patients (n=3). (*p<0.001, **p<0.05).

vation, which in turn serves as pathway for ROS and 8-iso-PGF2α formation.

Our findings are apparently in contrast with Walsh et al., who showed the presence of Nox1 in platelets using a specific Nox1 inhibitor (8). However, our western blot analysis could not confirm Nox1 expression in platelets. We are also in disagreement with Vara et al. (12) who did not find Nox5 expression in platelets; the different antibodies may perhaps account for this divergent results. A limitation of the study is in the use of a peptide which inhibits not only Nox5 but also platelet CAMKII, a protein involved in ROS formation (13).

In conclusion, we provide the first evidence that platelets from X-CGD patient express Nox5, which is likely to serve for ROS and isoprostane formation. The expression of Nox5 by platelets from X-CGD suggests Nox5 as reservoir enzyme for ROS formation.

Conflicts of interest
None declared.

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