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Detection of Free Radical Reaction Products and Activated Signalling Molecules as Biomarkers of Cell Damage in Human Keratinocytes upon Lead Exposure

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Abstract

Lead (Pb) is one of the most important environmental pollutant metals accumulating in the atmosphere, water, foods, and in organisms living in contaminated areas. Skin is one of the main targets of Pb toxicity based on its ability of direct penetration upon exposure. The underlying cell damaging pathomechanisms have not been revealed in detail. Herein, we focus on Pb-induced oxidative and nitrosative stress that has not been previously thoroughly investigated. We investigated these effects in order to elucidate the pathomechanisms and as well to identify potential biological markers that may indicate Pb toxicity. Human immortalized keratinocytes (HaCaT cells) were exposed to Pb (100 µM) either for 5 minutes or 6 hours. Pb-induced cellular damage was evaluated by immunocytochemistry analysis of multiple signalling cascades, e.g. apoptosis, Akt, MAPK, NOS, nitrotyrosine and 8-isoprostane formation, detection of nitrosative stress using Diaminofluorescein (DAF-FM) and oxidative stress using 3'-(p aminophenyl) fluorescein (APF). We found that Pb exposure resulted in significantly enhanced NO and ROS production in HaCaT cells. Pb led to enhanced eNOS-phosphorylation at Ser^{1177}, and Ser^{116} residues but not Thr^{495}. AKT phosphorylation but not MAP kinases were enhanced by Pb In addition, Pb induced apoptosis as shown by Caspase-3 activation and PARP cleavage. Our results suggest that Pb mediates its toxic effect in keratinocytes through oxidative and nitrosative stress which is accompanied by differential changes of eNOS phosphorylation and apoptosis. These data significantly contribute to understanding of underlying mechanisms of Pb-induced cellular damage.

Keywords: HaCaT; Lead; Nitrosative stress; Oxidative stress; Apoptosis

Introduction

Lead (Pb) is one of the most important metals that pollute the natural environment due to man's impact. As Pb cannot be degraded, it accumulates in the atmosphere, water, foods, and in organisms living in contaminated areas [1]. Environmental accumulation with Pb has accelerated due to its dose relationship to industrialization, major sources of lead exposure are dust, water, paint, cosmetics, folk remedies, and food supplements [2]. Pb causes haematological, gastrointestinal, and neurological dysfunction. Prolonged exposure to Pb may also cause reproductive impairment, hypertension, and nephropathy. Furthermore, Pb slows nerve conduction, alters calcium homeostasis, inhibits enzymes, and stimulates synthesis of binding proteins [3]. The persistence of Pb in animals and humans and the associated health risk is a highly relevant topic of current concern.

Many investigators have shown that Pb intoxication induced cellular damage mediated by formation of reactive oxygen species (ROS) [4,5]. Reactive oxygen species (ROS) have been proposed to play important roles in heavy metal-associated toxicity and pathology. The production of ROS including superoxide ion, hydrogen peroxide, and hydroxyl radical as mediated by heavy metals could further react very rapidly with DNA, lipids, and proteins, causing cellular damage. Although cells have elaborate homeostatic mechanisms to maintain intracellular redox equilibrium, persistent oxidation and the resulted interference with DNA replication have been implicated to be the major sources of endogenous DNA damage and genomic instability, both of which have been observed during early steps of human tumorigenesis [6-8].

Pb toxicity has been attributed in part to the disruption of calcium (Ca^{2+})-dependent mechanisms [9]. Pb modulates Ca^{2+} channels, Ca^{2+}-binding proteins and Ca^{2+}-dependent protein kinases [10], including protein kinase C [11] and Ca^{2+}/calmodulin-dependent protein kinase II [12].

Lin et al. 2003 [57] indicated that the aetiology of Pb genotoxicity is rather complex because of the reports that indicated that Pb exhibits weak genotoxicity in cultured rodent cells and does not cause mutations in human cells while other reports provided an *in vitro* evidence that this non-essential toxic metal can destabilize DNA helical structure and induce DNA strand breaks and oxidative DNA adducts.

Although Pb is not a transition metal, the catalysis of peroxidative reactions by Pb may be a major contributor to the toxic effects of this metal [13] have reviewed the role of oxidative tissue damage and altered fatty acid composition in the toxicity of Pb, and based on the evidence presented, oxidative mechanisms appear to be involved in some of the toxic effects of Pb. Dose- and time-dependent increases in peroxides in hepatic microsomal membranes and arachidonic acid content occur in response to Pb [14].

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As with some transition metals, such as cadmium, Pb results in an increase in glutathione levels in tissues including liver, kidney, and erythrocytes. This increase in tissue glutathione appears to be a compensatory response to ameliorate Pb toxicity [15].

One possible mechanism of Pb toxicity may be the interference with nitric oxide (NO) production, as it had been shown *in vitro* [16]. NO is a gaseous substance produced by the family of nitric oxide synthases (endothelial, eNOS; inducible, iNOS; neuronal, nNOS) from L-arginine. It was demonstrated that eNOS and iNOS are expressed and active in human skin [17]. As NO is an unstable and highly reactive compound with a short half-life, NOS activity is commonly measured reflecting NO levels. Pb affects NO production through inhibition of NOS activity [18]. Changes in NOS functions could result in a cascade of pathophysiological effects.

Previous aspects suggest that ROS detection or RNS formation, Pb evaluation of radical reaction products as well as Pb-induced changes of significant signalling cascades could indicate the degree of cell damage and contribute to clarify cellular mechanisms occurring upon Pb exposure. This applies in particular to skin and mucous membranes, which are directly exposed to environment pollutants, such as Pb. But possible hazardous effects of Pb on keratinocytes have not been thoroughly investigated. Therefore, we have herein explored Pb-induced alterations of ROS- and RNS-levels, influences of Pb on MAPK and Akt-Kinase pathways, changes of eNOS-phosphorylation and iNOS expression as well as Pb-induced apoptosis activation. Our results will pave the way to identify key biomarkers related to Pb exposure and to provide a deeper insight into Pb-related pathomechanisms in keratinocytes.

Material and methods

Cell culture

Spontaneously immortalized HaCaT cells were obtained from the German Cancer Research Center (Heidelberg, Germany [19]. Cells were grown in DMEM culture medium supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 U/ml) and were kept at 37°C in a humidified atmosphere of 5% CO_2. Cell cultures were split and sub-cultured as described for keratinocytes. HaCaTs were seeded for immunocytochemistry onto 0.1% gelatine pre-coated cover slips in 24-multiwell plate. The HaCaT cells were treated with 100 µM Pb [20] in culture medium for 5 minutes or 6 hours, and compared with control (sham-treated with H_2O as vehicle control).

Immunocytochemistry

The pretreated HaCaTs were kept in 4% paraformaldehyde for 25 min. and then rinsed several times with 0.1 M phosphate-buffered saline (PBS). For blocking unspecific binding sites, 5% BSA in Tris-buffered saline (TBS) was used (1 hour at room temperature). Prior to each step, cells were rinsed in TBS buffer for three times. Incubation with the primary antibody was performed in a TBS-based solution of 0.8 % BSA overnight at 4°C. The following antibodies were applied (for details see Material section): polyclonal rabbit anti-eNOS (1:400), anti-p-eNOS at Ser^{116} (1:500), anti-p-eNOS at Thr^{495} (1:400), anti-p-eNOS at Ser^{1177} (1:500), anti-AKT1/PKB (1:500), activated anti-Caspase-3 antibody (1:500), c-PARP (1:250), iNOS (1:1500), anti-3-nitrotyrosine (1:500) or 8-isoprostane (8-epi-prostaglandine F2 alpha (8-epi-PGF2a) (1:1500). After rinsing with TBS, the cells were incubated with the corresponding secondary biotinylated goat anti-rabbit IgG (1:400), biotinylated goat anti-mouse IgG (1:400) for 1 hour, followed by a streptavidin horseadish complex (1:150) for 1 hour. Finally, the antibody-staining was visualized by 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-HCl buffer and 0.1 % H_2O_2 for 5 minutes. Incubations without the primary antibodies were carried out as negative controls. To objectify the results, we measured the grey values of 100 cells from 3 independent experiments. The intensity of immunostaining was reported as the mean of measured HaCaT gray value minus background gray value (referred as densitometric units [DU]). The background gray value was detected at a cell free area of the cover slip. For staining intensity detection a Leica microcope (Wetzlar, Germany) coupled to a 3-chip CCD-camera (DXC-1850P, Sony, Germany) was used and the analysis was conducted using the software "ImageJ" (National Institutes of Health, Bethesda, Maryland, USA). Magnification for all images was 500-fold.

Detection of NO radical formation in HaCaT cells using DAF-FM DA

The formation of NO in HaCaT cells was assayed using 4,5-diaminofluorescein diacetate (DAF-FM diacetate), which is a cell membrane permeable form of the NO indicator. Once inside the cell, acetate moieties are cleaved by unspecific cellular esterases resulting in trapping DAF-FM within the cells. Whereas DAF-FM is an almost non-fluorescent compound, the reaction with NO forms a highly fluorescent benzotriazole. HaCaT cells were loaded with 10 µM DAF-FM DA for 10 minutes in a saline solution (containing in mmol l^{-1}: $CaCl_2$ 1.8, $MgCl_2$ 1, KCl 5.4, NaCl 137, NaH_2PO_4 0.2, Glucose 5.5, $NaHCO_3$ 12, L-arginine 1, pH 7.4) at 37°C. After washing with PBS, HaCaT cells were exposed to Pb or H_2O and DAF-FM fluorescence was imaged over time at 400-fold magnification with Ex 495 nm and Em 520 nm using a confocal laser-scanning microscope (LSM 510, Zeiss, Germany). Confocal images were digitally acquired and processed using the Meta 510 software (Zeiss, Germany).

Detection of reactive oxygen species in HaCaT cells using APF

3'-(p-aminophenyl) fluorescein (APF) is a non-fluorescent molecule until it reacts with either hydroxyl radicals, peroxynitrite anions ($ONOO^-$) or peroxy radicals. Cleavage of the aminophenyl ring from the fluorescein ring system results in bright fluorescence. APF was added to the media in a final concentration of 10 µM and cells were allowed to incubate for 30 minutes, washed, followed by Pb or H_2O (as control) exposure. Fluorescence was visualized over time at 400-fold magnification with Ex 490 nm and Em 520 nm using a confocal laser scanning microscope (LSM 510, Zeiss, Germany) equipped with a 490 nm excitation and 520 nm emission filters. All confocal images were analysed using Meta 510 software (Zeiss, Germany).

Materials

All chemicals were reagent-grade and if not indicated otherwise purchased from Merck (Darmstadt, Germany); Pb: lead-II-acetate-3-hydrate [$Pb(H_3CO_2)_2$] and anti-diphospho-ERK1/2 (# M 8159) were from Sigma (St. Louis, MO, USA). The NO-sensitive fluorescent dye 4,5-diaminofluorescein diacetate (DAF-FM DA) was from Alexis Biochemicals (Lörrach, Germany), APF (aminophenyl fluorescein: 2-[6-(4'-amino) phenoxy-3H-xanthene-3-one-9-yl] benzoic acid) was from Alexis Biochemicals (Lörach, Germany), The rabbit polyclonal phospho-specific antibodies recognizing Ser^{1177} (# 07-428), Ser^{116} (# 07-357), Thr^{495} eNOS, as well as rabbit polyclonal anti-Akt1/PKB (# 06-885) and anti-3-nitrotyrosine (#06-284) were purchased from Upstate

(Millipore) (Lake Placid, NY, USA). The anti-activated Caspase-3 antibody was a rabbit polyclonal antibody and purchased from BD (Pharmingen). The antibody against cleaved PARP (#G7341) was obtained from Promega (Mannheim, Germany). eNOS (#SA-201) and iNOS (#SA-200) antibodies were from Biomol (Hamburg, Germany). Anti-8-isoprostane (#IS 20) antibody was from Oxford Biomedical Research (Rochester Hills, MI), anti-JNK (#SC-474) antibody was obtained from Santa Cruz Biotechnology (Dallas, USA) and anti-phospho-p38 (#ab50012) antibody was from abcam (Cambridge, MA), respectively.

The secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-rabbit (#E0433) or HRP-conjugated rabbit anti-mouse IgG (#E0432) from Dako (Hamburg, Germany). The DMEM culture medium was obtained from GIBCO-BRL (Gaithersburg, MD), FCS was from PAA (Pasching, Austria), penicillin and streptomycin from Invitrogen (Karlsruhe, Germany).

Statistical analysis

All results are given as mean ± standard deviation (SD); n refers to the number of studied cells. Comparisons of mean gray values of the immunohistochemical analyses were performed by of one-way ANOVA. If the main effect was significant at level 5%, then pair wise contrasts were tested (correction of contrast *p*-values were not necessary). Repeated-measures ANOVA with covariance type AR (1) were applied to evaluate group (H_2O, Pb) differences in the mean values of several variables (DAF-FM fluorescence (intensity), APF fluorescence (intensity), i.e. Figures 1 and 2). If main effects of treatments were significant at level 5%, then pair wise contrasts between groups were tested (contrast *p*-values were Bonferroni corrected). Statistical differences were considered to be significant for values of $p<0.05$. Data analyses were performed by using SPSS 22 for Windows.

Results

Pb induced formation of radical stress (NO, ROS and RNS) and alteration of cellular signal transduction

Pb increased NO production: The intracellular production of free NO-radicals was measured by the NO-sensitive fluorescent dye (DAF-FM DA) during a 5 minutes period of BaP exposure. The measurement was started directly after adding Pb to the cells without any incubation time to avoid interference and measurement of endogenously produced NO independently of Pb. The DAF-FM fluorescence showed a time dependent significant increase in NO release after Pb exposure throughout the whole treatment period compared to the control (H2O) group, although it showed a fair acceleration throughout the whole period (Figure 1).

Pb increased ROS production: APF was used to detect the cellular ROS production upon exposure of HaCaT cells to Pb. An increased release of ROS was observed throughout the period of measurement (Figure 2).

Phosphorylation status of the different endothelial NO synthase (eNOS) residues: Treatment of HaCaT cells with Pb (100 µM) for 5 minutes revealed a significant increase of phospho-eNOS^{1177} expression (equivalent to bovine phospho-eNOS^{1179}) (H_2O: 36.53 ± 9.997 vs. Pb: 43.15 ± 12.52, $p<0.001$) (Figure 3A), and eNOS Ser^{116} (H_2O: 10.71 ± 5.37 vs. Pb: 12.88 ± 5.90, $p<0.019$) (Figure 3B), while total eNOS expression has hardly changed (H_2O: 9.61 ± 3.33vs. Pb: 10.63 ± 4.33, $p=0.062$). On the other hand, Pb downregulated the phoshorylation of eNOS Thr^{495} (H_2O: 11.45 ± 6.06 vs. Pb: 6.23 ± 2.70, $p<0.001$) (Figure 3C).

Influence of short-term (5 minutes) Pb exposure on Akt and MAPK (ERK, p38, JNK) phosphorylation: Treatment with Pb resulted in a significant increase in phosphorylation of AKT (H_2O: 4.79 ± 2.45vs. Pb: 5.99 ± 2.7, $p=0.043$), while AKT expression was

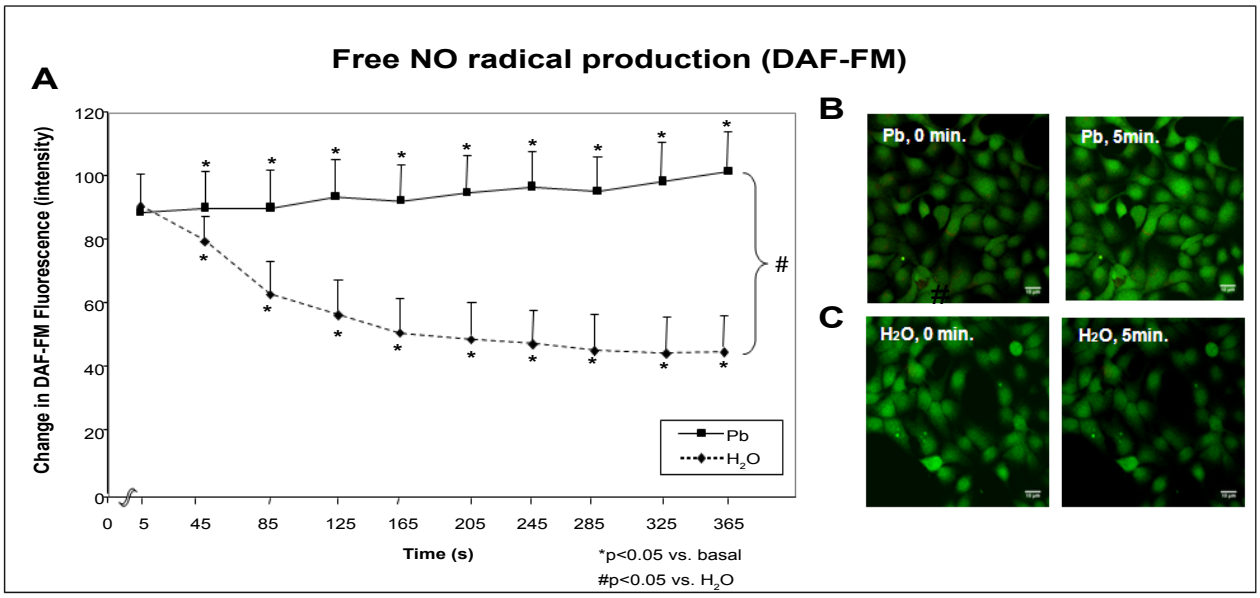

Figure 1: (A) Representative DAF registrations. The time dependence of the changes in DAF-FM fluorescence's was determined in HaCaT cells. The 5 minutes treatment with Pb led to a significantly increased DAF signal (*p<0.05). Finally, there was a time-dependent increase in NO release under treatment with Pb (n=25 cells per examination) in comparison to control (H_2O, n=25 cells per examination). Repeated measures ANOVA with covariance type AR were applied to evaluate group (H_2O, Pb) differences in the mean values of DAF-FM fluorescence (intensity). The main effects of treatments were significant at level 5%. The significant result (*p<0.05) was marked with asterisk. (B) Photomicrographs of the DAF-stained HaCaT cells under treatment with Pb at time point 0 and 360 seconds and of H2O treated controls (C) also at time point 0 and 360 seconds (Magnification=400 fold; Bars=100 µm).

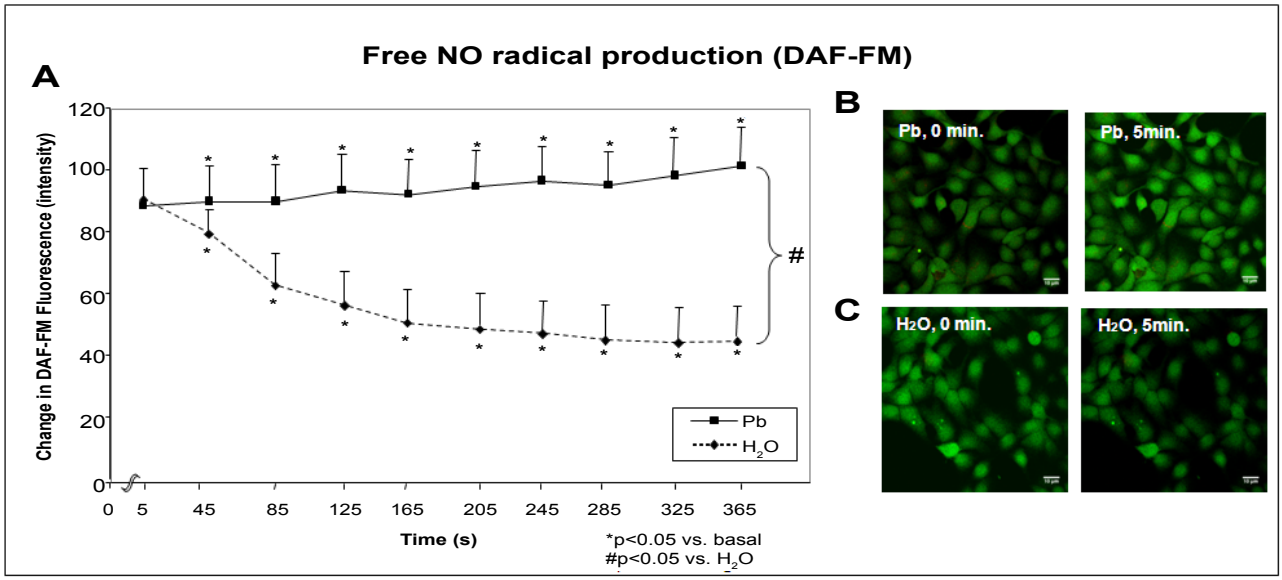

Figure 2: Pb-treatment induced RNS-reaction product. (A) Representative APF registrations. In both groups, the APF signal after 5 minutes treatment with Pb had significantly increased (*p<0.05) as a sign of induced RNS-reaction product accumulation. The main effects of treatments (Pb vs. H2O) were significant at level 5%. The significant result (*p<0.05) was marked with asterisk. (B) Photomicrographs of the ROS accumulation (APF-stained HaCat cells) under treatment with Pb at time point 0 and 360 seconds and of H2O treated controls (C) also at time point 0 and 360 seconds (Magnification=400 fold; Bars=10 µm).

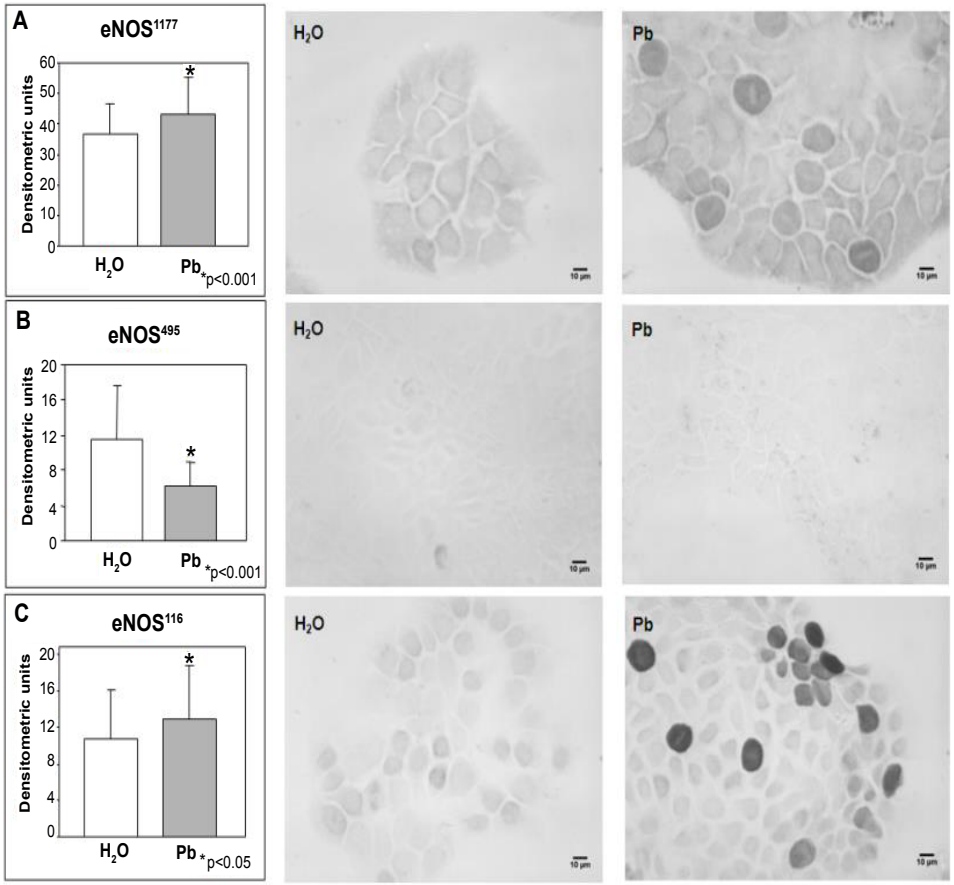

Figure 3: Phosphorylation status of the different endothelial NO synthase (eNOS) residues after treatment with Pb (100 µM) for 5 minutes. (A) Under the Pb treatment, there was a significant increased phosphorylation at serine 1177 residues (*p<0.05) of the eNOS. (B). The Pb treatment led to a significant decrease in the phosphorylation at threonine 495 residues (*p<0.05), (C) a significant increase in the phosphorylation at serine 116 residues (*p<0.05). For full data on results after Pb-treatment see supplement table 1. Values are presented as means ± SD. On the left side, photomicrographs of the immunocytochemical-stained cells are shown (Magnification=500 fold; Bars=10 µm).

unchanged, H_2O: 20.13 ±5.45 vs. Pb: 21.17 ± 4.67 (p>0.05).

Pb has almost no influence over the phosphorylation of ERK (H_2O: 8.03 ± 3.41 vs. Pb: 8.97 ± 4.16, p<0.082), as was the same case with ERK expression (H_2O: 12.50 ± 5.13 vs. Pb: 11.47 ± 5.80, p>0.05). On the other hand, both Phospho-p38 (H_2O: 10.60 ± 3.94 vs. Pb: 7.25 ± 3.02, p<0.0001), and JNK expression (H_2O: 18.43 ± 7.62 vs. Pb: 13.38 ± 5.93, p=0.00036) were downregulated by Pb.

Measurement of 3-nitrotyrosine and 8-isoprostane after 6 hours of Pb exposure: To confirm our results concerning the increased NO release after Pb exposure throughout the whole observation period measured by the NO-sensitive fluorescent dye (Figure 1), independent parallel experiments were conducted to assess 3-nitrotyrosine formation in cultured HaCaT cells upon exposure to Pb. Experiments showed a significant increase of 3-nitrotyrosine formation after 6 hours of Pb treatment (H_2O: 13.62 ± 4.13 vs. Pb: 15.01 ± 6.13, p<0.05) (Figure 4A).

8-Isoprostane is a stable end product of arachidonic acid oxidation by ROS and is therefore suitable as a marker for oxidative stress. We measured the formation of 8-isoprostane and found a significantly increased 8-isoprostane formation (H_2O: 7.93 ± 2.42 vs. Pb: 12.53 ± 5.34, p<0.001) (Figure 4B).

Signs of cellular damage

Pb decreased iNOS expression: NO, especially when produced by iNOS, is involved in multiple cellular regulation processes as well as in cytotoxic events. Therefore we examined the expression of iNOS after treatment with Pb for 6 hours. iNOS expression was significantly downregulated after 6 hours of Pb treatment (H_2O: 9.24 ± 4.52 vs. Pb: 7,067 ± 3,51, p<0.001) (Figure 5).

Influence of Pb on apoptosis: The induction of apoptosis is an important cellular response to DNA damage but also to other cell damaging agents. Therefore we examined both caspase-3 activation and PARP cleavage after Pb exposure for 6 hours. Pb has significantly increased activated Caspase-3 expression (H_2O: 6.29 ± 3.73 vs. Pb: 16.1 ± 6.49, p<0.001) (Figure 6A) and let to c-PARP cleavage (H_2O: 9.69 ± 4.79 vs. Pb: 12.45 ± 3,311, p<0.001) (Figure 6B) (Table 1).

Discussion

Pb induced formation of radical stress (NO, ROS and RNS) and alteration of cellular signal transduction

Pb increased NO production: Our results showed that Pb significantly increased NO production as was measured by means of DAF-FM, NO production was significantly increased with fair acceleration throughout the whole period of treatment compared to the control (H_2O). Sharifi et al 2005 [21], pointed out the significance of increased NO production by proposing that it could partly mediate the Pb-induced cytotoxicity. While clinical and experimental evidence is accumulating in support of an important role for Pb-induced oxidative stress and depressed nitric oxide (NO) availability [22-25]. Although

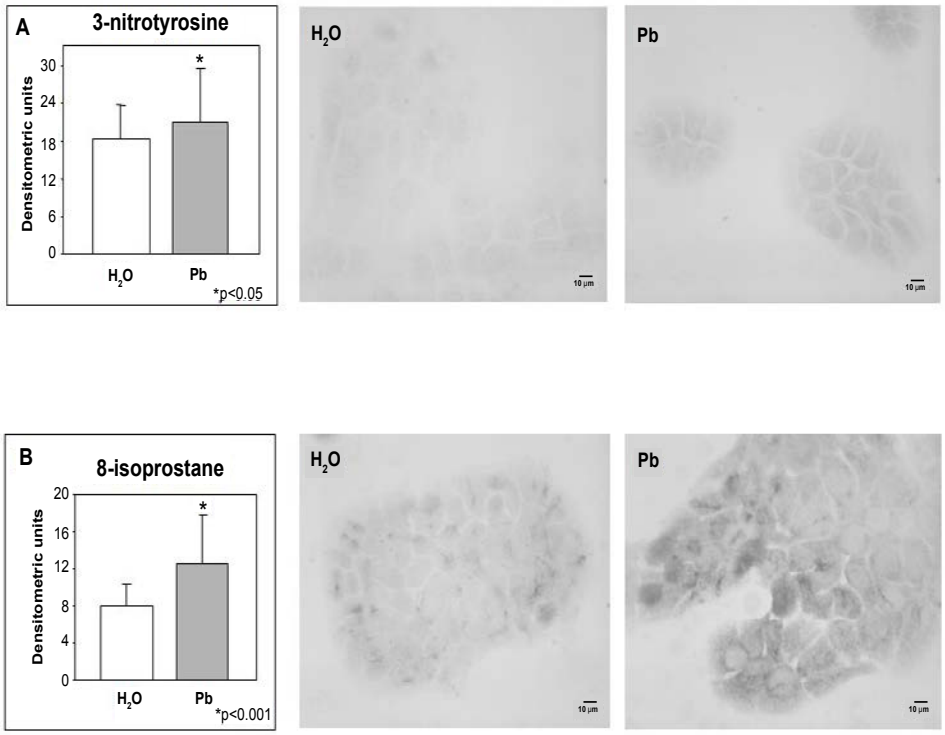

Figure 4: (A) Immunocytochemical detection of the Nitrotyrosine after 6 hours. The semiquantitative analysis of immunocytochemical staining of Nitrotyrosine in the Pb-treated cells (100 µM) compared to control (each group: n=100 cells). Comparisons of the mean gray values of the immunocytochemistry analyses were performed with the use of one-way ANOVA. The Pb treatment led to a significant increase of Nitrotyrosine, *p<0.05. (B) Immunocytochemical detection of 8-isoprostane after treatment with Pb (100 µM) for 6 hours. The plots of the arbitrary gray value of the immunocytochemical staining for 8-isoprostane, shows significant enhancement of this protein (p<0.0001) under the Pb treatment. Comparisons of the mean gray values of the immunocytochemistry analyses were performed with the use of one-way ANOVA. The left side of the figure shows exemplary photomicrographs of immunocytochemical-stained HaCaT cells exposed to 100 µM Pb or with H_2O (as solvent of Pb) for 8-isoprostane (Magnification=500 fold; Bars=10 µm).

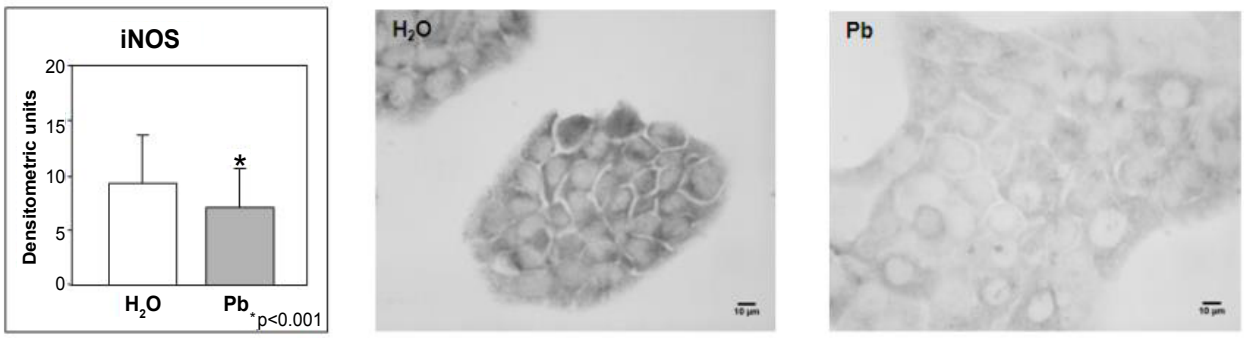

Figure 5: Immunocytochemical detection of iNOS after treatment with Pb for 6 hours. The graph of the arbitrary gray values of the immunocytochemical staining for iNOS after treatment with Pb showed a significant elevation of this protein (*p<0.05) after treatment with Pb. The increased iNOS expression appears to be a ROS-dependent process. The gray values of at least 100 HaCaT cells were identified per treatment group (i.e. n=100 cells for the control group (H_2O) and n=100 cells for the Pb group). Comparisons of mean gray values of the immunocytochemistry analyses were performed with the use of one-way ANOVA (Magnification=500 fold; Bars=10 µm).

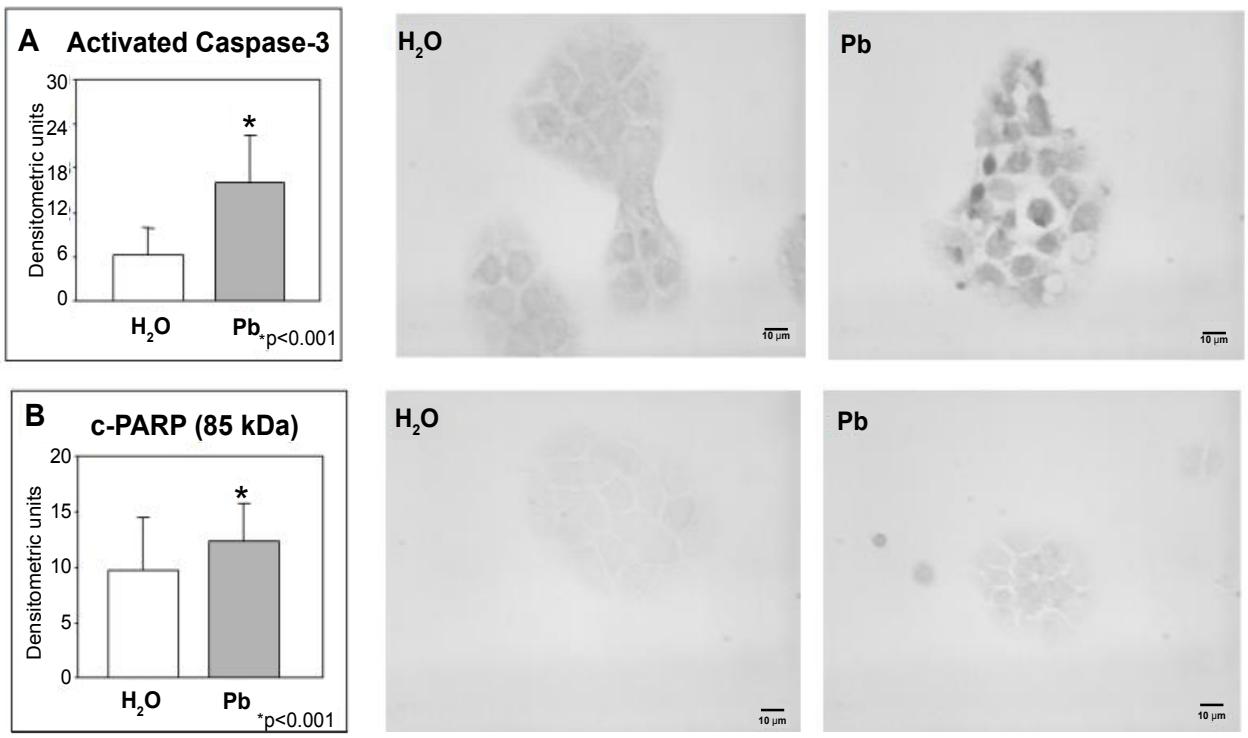

Figure 6: Pb-treatment stimulates apoptosis in HaCaT cells. Therefore cells were plated at 2-3x10^3/cm^2 onto glass cover slips. After 3 days, cultures were treated with standard medium supplemented with Pb (or H_2O) for 6 hours. For apoptosis, activated Caspase-3 was measured by immunocytochemistry. The immunocytochemical staining detected a significant increase of activated Caspase-3 (*p<0.05), i.e. the increased oxidative stress is socialized with an activation of Caspase-3 signalling pathway. Comparisons of mean gray values of the immunocytochemistry analyses were performed with the use of one-way ANOVA. Shown are the mean ± SD and are representative of at least 3 independent experiments with n=100 cells. (B). Under the Pb treatment, there was a significant increase in the 85 kDa PARP cleavage product (*p<0.05) as a sign of switching-on of apoptosis (Magnification=500 fold; Bars=10 µm).

exposure to Pb alone inhibits NO production *in vitro* in murine splenic macrophages [16], Pb combined with LPS increased nitric oxide (NO), NO-initiated oxidative stress, and TNF-α, all of which were involved in the development of hepatic injury in rats after they had been treated with Pb and LPS [26]. Such generation of free radicals can induce membrane lipid peroxidation and cause damage to proteins and nucleic acids, resulting in hepatic injury during oxidative stress [27]. On the contrary, [28] found that nitrite (oxidation products of NO) was significantly decreased in the culture media of aorta exposed to Pb.

Pb increased ROS production: In the present work, APF was used to detect the cellular ROS production upon exposure of HaCaT cells to Pb. We could observe an increased release of ROS was observed throughout the period of measurement.

Taking together, the fair NO production along with the increased ROS production, we could assume that NO production is quickly

Treatment	Control (H_2O)	Pb (100 µM in H_2O)	p-Value	↑↔↓
For 5 min				
eNOS	9.61 ± 3.33	10.63 ± 4.33	0.062	↔
peNOS116	10.71 ± 5.37	12.88 ± 5.90	0.019	↑
peNOS495	11.45 ± 6.06	6.23 ± 2.70	<0.001	↓
peNOS1177	36.53 ± 9.997	43.15 ± 12.52	<0.001	↑
ERK	12.50 ± 5.13	11.47 ± 5.80	0.350	↔
pERK	8.03 ± 3.41	8.97 ± 4.16	0.082	↔
AKT	20.13 ±5.45	21.17 ± 4,67	0.299	↔
pAKT	4.79 ± 2.45	5,99 ± 2,7	0.043	↑
pJNK	18.43 ± 7.62	13.38 ± 5.93	0.00036	↓
phospho-p38	10.60 ± 3.94	7.25 ± 3.02	<0.0001	↓
For 6 h				
activated Caspase-3	6.29 ± 3.73	16.1 ± 6.49	<0.001	↑
c-PARP (85 kDa)	9.69 ± 4.79	12.45 ± 3,311	<0.001	↑
iNOS	9.24 ± 4.52	7,067 ± 3,51	<0.001	↓
8-isoprostane	7.93 ± 2.42	12.53 ± 5.34	<0.001	↑
3-nitrotyrosine	13.62 ± 4.13	15.01 ± 6.13	<0.05	↑

Table 1: Overview of results after treatment with Pb, studies on Pb exposure (100 µM) on phosphorylation and apoptosis signaling cascade in HaCaT's using immunocytochemistry. The experiments were performed thrice with the antibody. In each series, the gray value of at least n=100 cells per group were measured. Shown are the means (given as mean ± standard deviation (SD)) as densitometric units (DU) for control vs. Pb. Statistical differences were considered to be significant for values of p<0.05.

deactivated. Thus, high ROS levels after Pb-exposure may increase the presence of superoxide anion, raising the probabilities of an interaction between NO and ROS to produce peroxynitrite, another highly deleterious molecule. Peroxynitrite is a strong, relatively long-lived oxidant which has been implicated in tissue injury. This compound is known to initiate lipid peroxidation, sulfhydryl oxidation and nitration of aromatic amino acids, such as tyrosine. Some of these actions might lead to irreversible tissue damage [29].

ROS are logical candidates to mediate NO modulation. Pb-induced oxidative stress contributes to the pathogenesis of Pb poisoning due to disrupting the delicate pro-oxidant/antioxidant balance that exists within mammalian cells [30]. It could be assumed that, it could be induced as a result of both depressed antioxidant system and increased ROS production [31]. It has been suggested that Pb-induced accumulation of H_2O_2 and O_2- could be through increasing the activity of NAD(P)H oxidase, glutathione reductase, superoxide dismutase and catalase [32].

Our findings are in line with [23], who declared that Pb administration induces ROS production that result in lipid peroxidation, DNA damage and depletion of cell antioxidant defense systems. This agrees as well with the previous reports that suggested that Pb was frequently associated with the blockade of NO generation and the increase in ROS formation in endothelial cells [33,34].

The association of oxidative stress with Pb intoxication suggests that an antioxidant may enhance the efficacy of therapeutic agents used in the treatment of Pb poisoning. The current approved treatment for Pb poisoning is to administerate chelating agents that form an insoluble complex with Pb and remove the same from Pb-burdened tissue. Moreover, administration of antioxidants during chelation therapy has been found to be beneficial in increasing Pb mobilization and providing recoveries in altered biochemical variables [35].

Phosphorylation status of the different endothelial NO synthase (eNOS) residues: Treatment of HaCaT cells with Pb for 5 minutes revealed a significant increase in phospho-eNOS^{1177} and phospho-Ser^{116} expression, while total eNOS expression has hardly changed. Pb reduced the phosphorylation of eNOS Thr^{495}.

The paradox between the fair level of NO and the upregulated expression of eNOS residues could be explained by suggesting that the NO was over-sequestrated under the influence of the reactive oxygen species (ROS), such as O_2^- which were markedly elevated in Pb-exposed animals [36] and *in vitro* cultured aorta [2,37].

Our findings are consistent with [38], who showed that under Pb treatment, constitutional NOS was inhibited (50% of the control), while iNOS activity was enhanced. Similar results were documented by [39], who reported low Pb concentration-induced changes of NO levels in cultured brain endothelial cells through its effect on constitutional NOS [40], have as well showed that Pb can inhibit constitutive NO synthase, which is Ca^{2+}-dependent. While the specific mechanism involved in this inhibition is not clear, it is hypothesized that Pb displaces Ca^{2+} from calmodulin, thus inhibiting the enzyme [41]. In addition, Pb exposure may also cause NO inactivation by increasing oxidative stress, thus decreasing NO availability [23-25].

Low NO production could partially be explained by the low total eNOS expression. This goes in line with [42] who reported that the eNOS protein mass in kidney cortex of Pb-treated rats was not significantly different from that of control rats. On the other hand [28], indicated that the expression of eNOS upon Pb exposure was significantly upregulated. Our result of eNOS expression partially agrees as well with some reports of animal studies where the eNOS protein mass was markedly raised both in aorta and kidney in response to Pb treatment [43].

The differential increase in the phosphorylation of eNOS residues, while eNOS expression was unchanged, and the minimal NO, but increased ROS production, might infer a decoupling of NOS function [41].

Zhu, et al. [45] might have explained the discrepancy in the NOS activity results throughout different researches by assuming that the degree of the inhibitory effect depends on the time span of exposure and the Pb concentration.

Influence of short-term (5 minutes) Pb exposure on Akt and MAPK (ERK, p38, JNK) phosphorylation: Treatment with Pb resulted in a significant increase in phosphorylation of AKT, while AKT expression was unchanged. Activation of AKT results in an increasing cellular proliferation and protection from apoptosis through phosphorylation and inactivation of several effectors including Bad, caspase-9, the forkhead family of transcription factors, GSK-3, p27 and p21 [46,47].

On the other hand, Pb has almost no influence over the phosphorylation of ERK as was the case with ERK expression, while both phospho-p38 and JNK expression were downregulated by Pb. Mitogen-activated protein kinases, a family of serine threonine kinases, are important signalling mediators of cellular stress response via regulation of different genes related to apoptosis. In earlier studies, it has been shown that p38 MAP kinase is a well-known member in stress-activated signal transduction [48,49] and ERK activation is strongly enhanced by over-expression of p38 [50] and mainly associated with cell survival and proliferation [51]. JNK2 was found to be critical for activation of the mitochondrial death pathway and mitochondrial translocation [52].

The MAPKs c-Jun-N-terminal kinase (JNK) and p38 are sensitive

to oxidative stress proposed to contribute to Pb toxicity [53-56]. On the other hand, [57] proved that Pb increased expression of phosphorylated ERK and AKT, but not phosphorylated p38 and JNK in human non-small cell lung adenocarcinoma CL3 cells. Due to the increased data discrepancy in the references about Pb ability to promote or inhibit the phosphorylation of the different members of the MAPKs, [57] came with the conclusion that the particular function regulated by MAPKs is likely to depend on the cell type, the stimulus and the duration and strength of kinase activities.

On the contrary, [20] showed that both p38 and JNK were significantly stimulated after treatment with Pb for 48 hours, yet ERK was not modified, they emphasized on the differential stimulation of the MAP kinases in different researches and how it is possibly related to the concentration and time of exposure of Pb. Our exposure time in that case was 5 minutes with a relatively small dose (100 μM) and that might explain in part that discrepancy.

Measurement of 3-nitrotyrosine and 8-isoprostane after 6 hours of Pb exposure: RNS are unstable molecules, but their formation can be estimated by the detection of nitrotyrosine protein components. Therefore, nitrotyrosine formation is accepted as a biochemical marker for ONOO- (peroxynitrite) formation [58,59]. In our experiments, we were able to observe a significant increase of nitrotyrosine formation after Pb exposure [43], pointed out that nitrotyrosine; the footprint of NO oxidation by ROS, was significantly increased in plasma, kidney, heart, liver, and brain of Pb exposed rats.

The results of [29] agree with our observations, where they showed that 3-nitrotyrosine abundance was higher in the Pb-treated rats. Taking together the increased nitrotyrosine, high ROS production and low NO production, we suggest a higher ROS production with reduced NO levels upon Pb exposure.

We were also able to demonstrate a significant increase in the formation of 8-isoprostane (the oxidized form of PGAα2), which is a biochemical indicator of oxidative stress [60]. This confirms our vital imaging results of ROS measurements.

Signs of cellular damage

Pb decreased iNOS expression: We were able to show that iNOS expression was significantly downregulated after 6 hours of Pb treatment. On the contrary, Vaziri et al., 1999 suggested that the increased reactive oxygen species (ROS) leads to reduced NO bioavailability and subsequently a compensatory upregulation of NO synthases (NOSs). But on the other hand, [44] suggested that there should be a balance between the NO production and degradation, so we assume that increased NO production could be accompanied with a positive feedback on iNOS decreasing its expression. Moreover [28], showed that there was no significant difference in iNOS expression in control and Pb-exposed group.

Influence of Pb on apoptosis: Our findings showed that Pb has significantly increased activated Caspase-3 along with its substrate c-PARP. This is in line with [61] who clearly indicated that Pb-induced apoptosis is caspase-mediated and accompanied by extracellular signal-regulated kinase (ERK) dephosphorylation.

Pb exposure activates caspases in the brain which suggests the proapoptotic effects of Pb. The way Pb induces caspase activation is not clear. The redox regulation of caspase activation [62,63] suggests that Pb may induce caspases through its pro-oxidant activity. Taken altogether, we conclude that Pb pathomechanisms in keratinoytes were mainly mediated through oxidative and nitrosative stress upon brief (5 minutes) and prolonged (6 hours) exposure. Differential activation of eNOS residues and cellular apoptosis could be mostly related to the AKT pathway activated by Pb, as we could show that the MAP kinases were either not affected or downregulated by Pb.

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