

Analysis of the signal transduction pathway of nickel-induced matrix metalloproteinase-2 expression in the human keratinocytes *in vitro*: preliminary findings

Background: Nickel can induce cellular and nuclear damages responsible for chronic diseases, like allergic contact dermatitis (ACD). We previously showed that matrix metalloproteinase-2 (MMP-2) gene expression was induced by nickel in nontumorigenic human keratinocytes cell line (HaCat).

Objective: To investigate the signal transduction pathways involved in gelatinolytic activity induced in HaCat under nickel stimulation.

Methods: We analyzed the involvement of protein kinase A (PKA), protein kinase C (PKC), tyrosine kinase (PTK), nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) using specific inhibitors (H89, calphostin C, genistein, carpain and curcumin) by electrophoretic mobility shift assay, reverse transcription-polymerase chain reaction and gelatin zymography.

Results: Our results indicate that nickel-induced MMP-2 production was inhibited with PTK, PKC and AP-1 specific inhibitors. Moreover, both PKA and NF- κ B were not involved in nickel pathway.

Conclusions: Using HaCat, we showed that curcumin and genistein can revert nickel-induced MMP-2 upregulation. Whether the use of PTK and AP-1 inhibitors has therapeutic ramifications in the management of ACD remains to be investigated.

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Nickel is a transition metal used in a variety of industrial processes. It is involved in allergic contact dermatitis (ACD), a condition associated with both acute and chronic damage of the skin.

The morbidity of ACD depends on its cause and the possibility of avoiding repeated or continued exposure. Clinically, ACD is more severe and acute in onset than irritant contact dermatitis (ICD) and is often pruritic. Patients can present acute (bright red

skin, edema and vesicle), subacute (less edema and erythema, minimal vesiculation or serous drainage, and excoriation) and chronic manifestations (scaling, fissuring, lichenification, mild erythema and excoriation). Patients may improve by avoiding allergens such as rubber or nickel.^{1,2}

The mechanism of nickel toxicity has not been fully characterized.^{3,4} CD4+ and CD8+ nickel-specific T lymphocytes have been isolated from

allergic patients. Interestingly, T regulatory 1 (Tr1) lymphocytes exist in nonallergic donors, limiting the onset of the immune response.⁵ Human skin equivalent cultures (epidermal keratinocytes and dermal fibroblasts) respond to nickel producing IL-1 α and TNF- α as primary alarm signals, which trigger the release of secondary chemokines (CCL20 and CXCL8).⁶ These molecules contribute to the intricate chemokine cascade, which is responsible for the trafficking of immune cells to the site of inflammation *in vivo*.

Nickel exposure induces several types of cellular and nuclear damage.^{7,8} Literature supports the hypothesis that nickel-induced toxicity is caused by an alteration in the gene expression rather than direct DNA damage.^{9,10} For example, transcriptional factors, matrix metalloproteinases (MMPs) and heat shock proteins can be induced by exposure to nickel.^{11,12}

MMPs represent a family of major matrix degrading Ca⁺⁺- and Zn⁺⁺-dependent enzymes. They are synthesized as secreted or transmembrane proenzymes and processed to an active form by the removal of an amino-terminal propeptide. Activation can be induced either by chaotropic agents or by cleavage by members of the MMPs family or other proteases.¹³ Tissue inhibitors of matrix metalloproteinases (TIMPs) are the major cellular inhibitors of MMPs.¹⁴ MMPs are divided into subfamilies, according to the substrate specificity.

MMP-2 (gelatinase A/M 72,00 type IV collagenase) and MMP-9 (gelatinase B/M 92,00 type IV collagenase) are key enzymes for degrading type IV collagen, which is a major component of the basement membrane.^{15,16} MMP-2 and MMP-9 seem to play roles in the alterations of keratinocytes and development of ACD *in vivo*.¹⁷

We have previously shown that nickel sulfate induced overexpression of MMP-2 messenger RNA (mRNA) and has a gelatinolytic activity using a keratinocyte cell line (HaCat) *in vitro*.¹⁸ During a skin pathological event involving an overexpression of MMPs, there is a consequent overexpression of angiogenic factors such as vascular endothelial growth factor.^{19,20} Multiple second messenger systems such as protein kinase, tyrosine kinase (PTK), mitogen-activated protein kinases appear to be related to the induction of MMPs in different experimental models.^{16,21–23} Moreover, activator protein-1 (AP-1) and nuclear factor-kB (NF-kB) have been investigated in cellular response to nickel compounds.²⁴ AP-1 is a transcriptional factor complex composed of Jun family homodimers or Jun/Fos heterodimers,^{25,26} and is involved in neoplastic transformations.^{27,28}

The aim of this study was to investigate the signal transduction pathways involved in gelatinolytic

activity induced in human keratinocytes, under nickel sulfate stimulation. We analyzed the involvement of protein kinase A (PKA), protein kinase C (PKC) and PTK in this process using specific inhibitors H89, calphostin C and genistein. Moreover, we investigated the potential involvement of AP-1 and NF-kB in nickel induction of MMP-2 expression. Two specific inhibitors were used (curcumin and carpain) to verify AP-1 and/or NF-kB involvement, respectively.

Materials and methods

Cell culture and treatment

The HaCat cell line, a spontaneously transformed nontumorigenic human keratinocyte cell line, was kindly provided by Professor N. E. Fusenig (Heidelberg, Germany). Cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) with 10% Foetal Calf Serum (FCS), 1% pen-strep and 1% glutamine (Gibco, Milan, Italy). Before treatment, cells were starved by serum deprivation for 24 h, in order to prevent selective activation of MMPs.^{22,18} Before metal stimulation, HaCat cells were treated for 1 h with H89 (1 μ M), calphostin C (300 nM) or genistein (40 μ M). Nickel sulfate (10⁻⁵ M) was added and cells were incubated for different lengths of time (24 h and 3 and 6 days), at 37°C in presence of 5% CO₂. Time intervals were selected as the most representative, based on our previous results.¹⁸ For AP-1 and NF-kB inhibition studies, cells were pretreated for 15 min at 37°C with curcumin 10 μ M or carpain 30 μ M, respectively.

Gelatin zymography

Gelatinolytic activity was assayed using the Heussen and Dowdle²⁹ method, adapted for a minigel format. Twenty-microliter aliquots of supernatants were applied directly without prior heating or reduction, to 10% (w/v) acrylamide gels containing 2 mg/ml of gelatin (Sigma, Milan). After removal of Sodium dodecyl sulphate (SDS) from the gel by incubation in 2.5% (v/v) Triton X-100 for 30 min, the gels were incubated at 37°C/18 h under continuous stirring in 50 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl, 5 mM CaCl₂ and 0.02% (w/v) Brij-35. Gels were stained for 30 min in coomassie brilliant blue diluted in 30% methanol/10% glacial acetic acid 0.5% (w/v) and then destained in the same solution without the dye. The gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gel. The intensity of the bands was quantified with specific densitometry (Molecular Analyst Software; Bio-Rad, Hercules, CA, USA).

Semiquantitative reverse transcription-polymerase chain reaction

RNA was purified from 1×10^6 HaCat cells using TRIzol reagent (Invitrogen SRL, Italy), according to the manufacturer's instructions. Briefly, cells were lysed directly in the culture dish using 1 ml TRIzol followed by the addition of chloroform. Centrifugation at 12,000g for 15 min at 4°C separated a colorless upper aqueous phase containing RNA. Finally, the RNA was precipitated by mixing with isopropyl alcohol. Total RNA was resuspended in 50 µl autoclaved diethyl pyrocarbonate-treated water (Sigma-Aldrich, Italy) and incubated for 30 min at 37°C with 7 U/µl DNase I ribonuclease free (Amersham Biosciences, Europe GmbH). Contaminating DNA was removed using MicroSpin™ S-400 HR columns (Amersham Biosciences, Europe GmbH, Berlin). The eluate containing RNA was analyzed on 1% agarose gel electrophoresis. The concentration was determined from absorbance at 260 nm and purity assessed by OD₂₆₀/OD₂₈₀ (optical density) ratio. Total RNA (0.4 µg) was used to perform complementary DNA (cDNA) synthesis using avian myeloblastosis virus reverse transcriptase (Promega Corporation, CA, USA) in presence of random hexamers (Amersham Biosciences, Europe GmbH).

MMP-2-specific primers were designed from the published cDNA sequences of the human MMP gene and synthesized by MWG-Biotech (Florence, Italy).¹⁸ Primers for TIMPs were synthesized as previously reported.³⁰ A summary of primer sequences used in this study is reported in Table 1. Control of the specificity of reverse transcription-polymerase chain reaction (RT-PCR) end products was based on the resolution of their molecular weight using gel electrophoresis. An aliquot (3 µl) of reverse transcriptase reaction mixture was used with primers (15 µM each), 2.5 mM desoxynucleoside triphosphate mixture (Amersham Biosciences, Europe GmbH) and 5 U/µl Taq DNA polymerase (Promega Corporation) in a total volume of 50 µl. Hypoxanthine phosphoribosyltransferase (HPRT)

cDNA was amplified to serve as a calibrator. The thermocycling profile of the PCR amplification consisted of denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 57°C for 1 min and 72 °C for 1 min. The number of cycles was predetermined to fall within the linear range of amplification of each PCR product. Amplified fragments were separated on a 2% agarose gel stained with ethidium bromide (10 mg/ml) and photographed.

Semiquantitative evaluations of the respective mRNA expression levels were performed by densitometry in comparison to those of the housekeeping gene HPRT (Molecular Analyst Software; Bio-Rad).¹⁸

Electrophoretic mobility shift assay

HaCat cells, unstimulated and stimulated with nickel in the presence of curcumin, were centrifuged and washed once in cold phosphate buffered solution (PBS). Cells were pelleted and nuclear extracts prepared from cells using the Andrews and Faller³¹ method and incubated with 70,000 c.p.m. of a 21-bp oligonucleotide containing AP-1 (c-jun) consensus sequence (5' CGCTTGATGAGTCAGCCG-GAA3') (Roche Diagnostics, Italy). AP-1 was detected using [α -³²P] d Adenosine triphosphate (dATP)-labeled IgK oligo probe. The DNA-binding reaction was performed with 20 ml of binding buffer [2 mg of poly (dI-dC) (Roche Diagnostics)], and the incubation was performed for 20 min at 25°C in the presence of 10 mM Tris-HCl, 1 mM MgCl₂, 50 mM NaCl, 0.5 mM ethylenediamine-tetracetic acid (EDTA), 4% glycerol and 0.5 mM dithiothreitol. For the competition experiments, a 100- to 200-fold molar excess of AP-1 and NF-kB cold consensus oligonucleotide was added to the binding reaction mixture prior to adding the probe and incubated for 10 min at 25°C.

The reaction product was analyzed via 4% polyacrylamide gel using 0.5X Tris-Borate-EDTA and electrophoresed for 2 h at 200 V. The gels were dried and exposed to X-ray film (Kodak) at -80°C using an intensifying screen.

Table 1. Primer sequences used for polymerase chain reaction amplification

Primer	Sequences	Product length (bp)
MMP-2	5'-TGACGGTAAGGACGGACTC-3' 3'-TGGAAGCGGATTGGAAACT-5'	342
TIMP-1	5'-TGACACCTGTGTCCACCCACCCACAGACG-3' 3'-TGGACCGTCAGGACGCCAGGGTCTATCGG-5'	551
TIMP-2	5'-CCGAATTCTGCAGCTGCTCCCGGTGCACCCG-3' 3'-GAGCTGTAGCTCCTGGGTATTTTCAAGG-5'	590
HPRT	5'-CCTGCTGGATTACATCCAAGCACTG-3' 3'-CCTGAAGTATTATTATAGTCAAGG-5'	369

MMP-2, matrix metalloproteinase-2; TIMP, tissue inhibitors of matrix metalloproteinases; HPRT, hypoxanthine phosphoribosyltransferase.

Statistical analysis

Experiments were run in duplicate and each experiment was repeated three times. Results were expressed as mean $\pm$ standard error of mean. Statistical analysis was performed by the Student's *t* test using SigmaStat software (SigmaStat for Windows version 3.1, Systat Software Inc. USA). Results were compared either with control samples (untreated) or nickel-treated samples, and means were considered significant if *p* < 0.05.

Results

Semiquantitative RT-PCR for MMP-2, TIMP-1 and TIMP-2. MMP-2, TIMP-1 and TIMP-2 mRNAs were upregulated by nickel contact for 24 h. Calphostin pretreatment reduced nickel-induced mRNA upregulation. Genistein suppressed MMP-2 and TIMP-1 expression below control levels, acting also on TIMP-2. H89 showed a strong reduction of TIMP-2 but had only a modest effect on MMP-2 and TIMP-1 mRNAs. (Fig. 1A). Only the inhibitors did not affect cell behavior (Fig. 1B).

Effect of inhibitors on MMP-2 expression by zymography

HaCat cells were treated with specific inhibitors, H89, calphostin and genistein for 1 h before nickel stimulation. Curcumin (10 μ M) and carpain (30 μ M) were incubated only for 15 min before adding nickel. Figure 2A shows the results obtained at

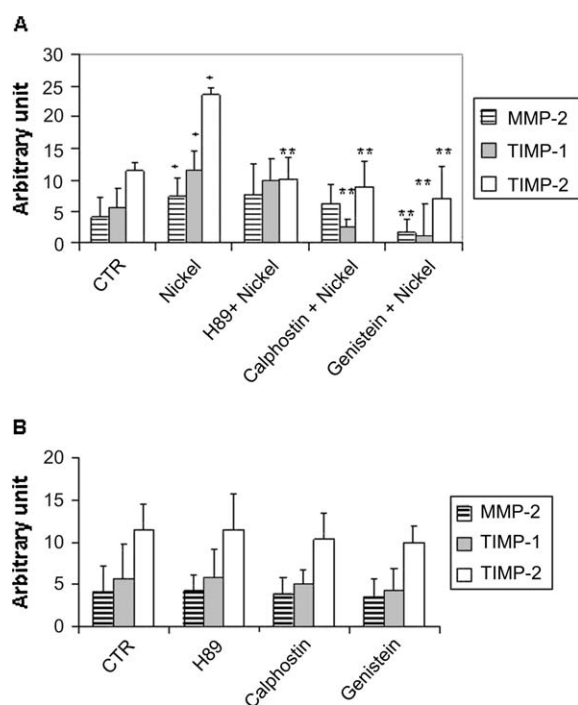


Fig. 1. Semiquantitative reverse transcription-polymerase chain reaction of metalloproteinase-2 (MMP-2) and tissue inhibitors of matrix metalloproteinases (TIMPs) messenger RNA (mRNA) in treated vs. untreated cells. A) Cells were stimulated with nickel sulfate 10^{-5} M for 24 h, with or without specific inhibitor pretreatment for 1 h with H89 (1 μ M), calphostin C (300 nM) and genistein (40 μ M). B) Effects of inhibitors alone in the same experimental conditions. Total RNA was extracted and amplified with specific primers. Levels of specific mRNA are reported as arbitrary units in comparison to the expression of the housekeeping gene. Results are expressed in arbitrary units. Values represent the mean $\pm$ standard error of mean of three independent experiments ($n = 6$). * $p < 0.05$ when compared to control (CTR) and ** $p < 0.05$ when compared to nickel-treated cells.

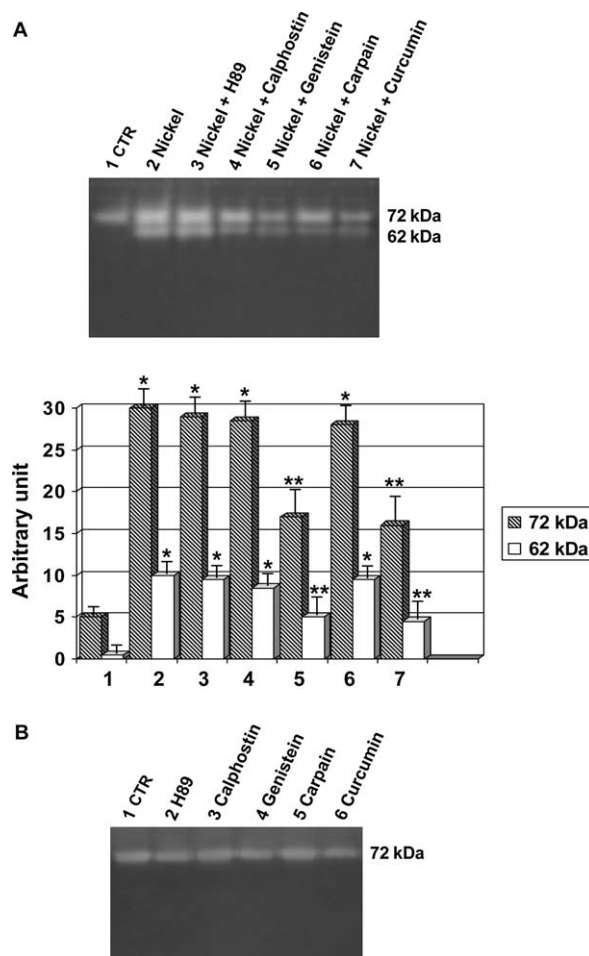


Fig. 2. Gelatin zymography for metalloproteinase-2 expression of treated vs. untreated HaCat cells. A) The cells were stimulated for 3 days with nickel sulfate 10^{-5} M, either alone or in presence of H89 (1 μ M), calphostin C (300 nM), genistein (40 μ M), carpain (30 μ M) and curcumin (10 μ M). B) Effects of inhibitors alone in the same experimental conditions. Results are expressed in arbitrary densitometric units. Values represent the mean $\pm$ standard error of mean of three independent experiments ($n = 6$). * $p < 0.05$ when compared to control (CTR) and ** $p < 0.05$ when compared to nickel-treated cells.

3 days of nickel contact. Longer treatment did not change the results obtained (up to 6 days). At that time, nickel sulfate (10^{-5} M) upregulated the MMP-2 72 kDa form compared to the control cells, and the active form of 62 kDa appeared. Pretreatment with H89 had no effect on the MMP-2 expression induced by nickel sulfate. Calphostin reduced both proenzymatic and active forms but the most efficient inhibition was caused by genistein. Carpain had no effect on MMP-2 expression and curcumin drastically reduced the MMP-2 expression. Figure 2B shows the results obtained using the inhibitors alone.

EMSA for AP-1

AP-1 activity was confirmed by the mobility shift assay after 24 h of treatment. The results obtained

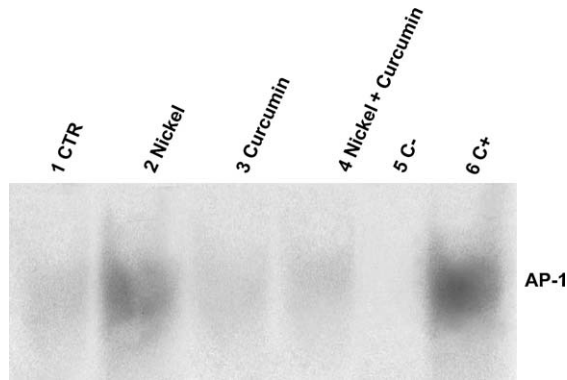


Fig. 3. Electrophoretic mobility shift assay with activator protein-1 (AP-1) consensus oligonucleotide in HaCat cells treated with nickel sulfate 10^{-5} M. Lane 1, untreated human HaCat; lane 2, HaCat treated with nickel sulfate for 24 h; lane 3, HaCat treated with curcumin; lane 4, HaCat pretreated with curcumin for 1 h, then exposed to nickel sulfate for 24 h; lane 5 (C-), binding reaction with unlabeled excesses of AP-1 consensus oligonucleotide; lane 6 (C+), binding reaction with excesses of nuclear factor-kB consensus oligonucleotide.

are shown in Fig. 3. Nickel sulfate induced a strong activation of AP-1 (lane 2) compared to the control cells, which showed only a basal level of AP-1 activity (lane 1). Curcumin (10 μ M), a specific AP-1 inhibitor, abolished AP-1 activity (lane 3) even in the presence of nickel stimulation (lane 4). Lane 5 showed the binding reaction with unlabeled excesses of AP-1 consensus oligonucleotide, while lane 6 showed the binding reaction with unlabeled excesses of NF-kB consensus oligonucleotide.

Discussion

A variety of soluble mediators such as cytokines, growth factors, adhesion molecules and MMPs are regulated by changes in gene expression in target cells during inflammatory process of the skin such as chronic ICD or ACD. The mechanisms of the development of chronic skin disease are not completely understood. The integrity of the skin depends on the attachment of its keratinocytes to the extracellular matrix (ECM) and to each other. The complex inflammatory process involves re-epithelization, connective tissue cell activation with subsequent ECM degradation and resynthesis.³²

ACD is characterized by inflammatory infiltrates, intercellular edema, leading to spongiotic vesicles and loss of cell-cell contact.¹⁷ Keratinocytes are programmed to maintain integrity and are the first cell to respond to injury, nickel being one of the most responsible among the sensitizing agents. Keratinocytes play a central role contributing to the local reaction through the expression of intercellular adhesion molecule (ICAM-1), and producing cytokines such as IL-1 β , TNF- α ,³³ IL-8 and MMP-2.^{18,34}

In this study, we investigated the pathways used by nickel to stimulate human keratinocytes cell line (HaCat) to enhance MMP-2 mRNA expression. Our results can help to characterize the physiological abnormalities leading to aberrant skin repair in acquired or inherited wound healing disorders.

HaCat cells were selected because they have been shown to be responsive to nickel exposure, similar to primary cultures of human keratinocytes,³⁵ and dose and time intervals were selected based on our previous work.¹⁸ In fact, we have shown that low concentrations of nickel sulfate (10^{-5} M) are able to stimulate type IV collagenase secretion within 3 days of contact and that the MMPs are reliable biomarkers of nickel exposure in HaCat cell lines.¹⁸

Type IV collagenases, MMP-2 and MMP-9, share structural and catalytic similarities but their responses to regulatory signals are different.^{36,37} The regulation of each MMP seems to be associated with different stages of differentiation, MMP-9 occurring in less differentiated cells, while the synthesis of MMP-2 is optimized at intermediate stages of differentiation. Moreover, MMP-2 synthesis seems to occur as a late event after lipopolysaccharide (LPS) stimulation.²⁴ The late induction suggests a role for MMP-2 in chronic inflammatory processes such as ACD. It was also reported that MMP-2 plays a role in the extravasation of T lymphocytes, suggesting the possibility that the augmented expression of MMP-2 in the chronic phase is involved in controlling the induction of allergic swelling reaction through the infiltration of T lymphocytes.³⁷ Although the regulatory pattern of MMP biosynthesis has been extensively studied, the intracellular signaling events controlling such regulatory patterns are not well understood. Different signaling pathways have been shown to be involved in the regulation of the MMPs expression, such as NF-kB, Cox-2, PTK, PKA, PKC, AP-1 and others in different experimental models.^{25,38-42} The promoter region of many MMP genes is highly conserved, with the common presence of a proximal (approximately -70 bp) AP-1-binding site.^{43,44} These conserved binding sites mediate many of the enhanced transcriptional responses of these genes to a variety of stimuli. Recently, some authors have shown that a functional AP-1 site mediates MMP-2 transcription⁴⁵ and that some molecules mediate MMP-2 production through the activation of AP-1.^{46,47} In a preliminary way, using different inhibitors, we have shown that MMP-2 production induced by nickel sulfate involves both PTK and PKC, but not PKA, and AP-1, but not NF-kB. The preclusion of PKA suggests that the MMP-2 production may not be mediated through the prostaglandin E2-dependent pathway as is the case for interstitial collagenase.⁴⁸ The involvement of PTK

and/or PKC can be explained by the presence of a common intermediary molecule such as phospholipase C- β , whose activation is dependent on tyrosine phosphorylation by membrane PTKs.⁴⁹ Tyrosine phosphorylation may be an early, critical event and a requisite for activating PKC. AP-1 is a nuclear target of PKC-mediated signal transduction in the skin.

Based on these preliminary findings, it is determined that the inhibition of PTK and/or AP-1 may be efficient in suppressing nickel-induced inflammatory disease of the skin.

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