HO-1 and VEGF gene expressions are time dependant during exposure to welding fumes


The Institute of Pulmonary and Allergic Diseases, National Laboratory Service for ILD, Tel-Aviv Sourasky Medical Center, 6 Weizman Street, Tel-Aviv 64239, Israel
Occupational Health Department, Israel Air Force, Israel
Israel Occupational and Preventive Medicine, Clalit Medical Services, Israel
Department of Epidemiology and Preventive Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Israel
Occupational Health Department, Israel Defense Forces, Israel

1. Introduction

Welding fumes can cause a variety of adverse health effects, from minor symptoms, such as headache, nausea, and metal fume fever, to severe ones, such as occupational asthma, bronchitis, pneumoconiosis, and lung cancer. Certain metal and gas components generated in welding fumes have been linked with inflammation and oxidative stress [1], and Cr, Ni, and Fe have been shown to produce reactive oxygen species (ROS), such as hydroxyl radicals (OH), superoxide anion (O2−), and H2O2 [2].

Increased production of ROS can trigger several key signaling events, which can provoke cell infiltration to the lung [2,3]. Those cells can respond to noxious fumes by biochemical and molecular changes, leading to disease [4]. Although epidemiological studies have demonstrated an increase in pulmonary illness after exposure to welding fumes [1], there is little information on the possible underlying mechanisms involved in pathology subsequent to the inhalation of welding fumes. Oxidative stress is caused by an imbalance between the production of ROS and a biological system's ability to promptly detoxify the ROS intermediates or easily and efficiently repair the resulting damage [4]. Heme oxygenase-1 (HO-1) is one of the central detoxifying pathways [5] whose metabolic pathway was first characterized by Tenhunen et al. [6]. It was suggested that HO-1 acts as an inducible defense against oxidative stress in models of inflammation, ischemia-reperfusion, hypoxia, and hyperoxia-mediated injury [7]. HO-1 is a microsomal enzyme which catalyzes the conversion of heme into CO and biliverdin. Those products together with the enzyme activity lead to the reduction of oxidation and inflammation [8]. The first known human case of HO-1 deficiency was presented in Japan in 1998 [9].

Vascular endothelial growth factor (VEGF) is a potent cytokine which promotes angiogenesis and mediates vascular permeability [10]. VEGF is reportedly essential for tissue rehabilitation on the one hand [11], while it is secreted during the inflammatory process and cancer on the other [12]. There are many publications in the literature that address the issues of respiratory effects of welding [1,2,13–14]. One recent
study used the methodology of sputum induction to study inflammatory change in welders [15]. We had previously shown that airway inflammation is present in asymptomatic welders, and that the particle burden, inflammatory cells, and level of oxidative stress are a function of the type and the duration of welding [16]. We now assessed the roles of HO-1 and VEGF genes in relation to the length of exposure to welding fumes and the particulate matter present in induced sputum (IS) samples of these asymptomatic workers.

2. Patients and methods

2.1. Patients

Welders were recruited from two aircraft plants and divided into two groups based on the exposure duration. The Welder 1 group was comprised of 30 males (mean age 36 ± 11 years) with short-term exposure (10.3 ± 10.8 years) to aluminum and iron that involved the use of an interface layer consisting of aluminum-rich brittle inter-metallic compounds (FeAl3 and Fe2Al5), and the Welder 2 group consisted of 16 males (mean age 50 ± 9 years) with long-term exposure (21 ± 7.6 years) to mild steel core often melting two pieces of metal and adding a filler substance containing chromium, iron, lead, and manganese to form a pool of molten material. The control group included 27 individuals (mean age 35 ± 10 years) working in administrative positions of the same facilities and who had no history of occupational exposure (Table 1).

Ethical approval was granted by the Tel-Aviv Sourasky Medical Center Institutional Ethics Committee. All the subjects gave written informed consent and made one clinic visit for clinical assessment, pulmonary function testing (PFT) and induction of sputum at the end of each working week. They were all asymptomatic and none had any history of respiratory or allergic conditions.

All the participants completed a demographic and clinical questionnaire on smoking habits, self-reported diseases, respiratory symptoms and years of exposure to welding fumes. Lung function was recorded with Masterlab body plethysmograph and spirometer (Masterlab E. Jaeger, Wurzburg, Germany). Measurements were made according to standard protocols of American Thoracic Society guidelines [17].

2.2. Induced sputum (IS)

Sputum induction was conducted in all three groups according to a standard method as previously described [18]. Briefly, nebulized 3% saline was administered through an ultrasonic nebulizer which the subjects inhaled for 15 min. The selected plugs were then the end of each working week. They were all asymptomatic and none had any history of respiratory or allergic conditions.

All the participants completed a demographic and clinical questionnaire on smoking habits, self-reported diseases, respiratory symptoms and years of exposure to welding fumes. Lung function was recorded with Masterlab body plethysmograph and spirometer (Masterlab E. Jaeger, Wurzburg, Germany). Measurements were made according to standard protocols of American Thoracic Society guidelines [17].

Table 1

Demographic, clinical and functional parameters of all participants (n = 73).a,b

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Age (year)</th>
<th>Smoking (no)</th>
<th>Exposure (year)</th>
<th>FVC (%)</th>
<th>FEV1/FVC</th>
<th>TLC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welders 1 (n = 30)</td>
<td>36 ± 11</td>
<td>23/30 (77%)</td>
<td>10.3 ± 10.8</td>
<td>95 ± 11</td>
<td>85 ± 6</td>
<td>91 ± 10</td>
</tr>
<tr>
<td>Welders 2 (n = 16)</td>
<td>50 ± 9</td>
<td>14/16 (87%)</td>
<td>21 ± 7.6</td>
<td>95 ± 16</td>
<td>82 ± 6</td>
<td>98 ± 17</td>
</tr>
<tr>
<td>Controls (n = 27)</td>
<td>35 ± 10</td>
<td>26/27 (93%)</td>
<td>—</td>
<td>95 ± 14</td>
<td>85 ± 7.7</td>
<td>97 ± 14</td>
</tr>
</tbody>
</table>

| P-value | 0.0001b | 0.063b | 0.002b | 0.87 | 0.23 | 0.12 |

PFTs were performed by a Masterlab spirometer.

Welders 1, short-term exposure to aluminum/iron; Welders 2, long-term exposure to cadmium/chromium/iron/nickel; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; TLC, total lung capacity; DLCO, diffusion lung CO; DLCO-SB, diffusion lung CO single breath; L, liters.

a The same patients reported in Tables 1 and 3 of our previous study [16].

b Analysis of variance (Kruskall–Wallis); a P < 0.05 was considered significant.

2.3. RNA extraction and real-time polymerase chain reaction (RT-PCR)

RNA extraction was done on the sputum cell by TRI reagent-chloroform solutions. Quantization of the RNA amount was done by GeneQuant™ 1300 Spectrophotometer (Amersham Biosciences). RT-PCR was carried out with 0.5 μg of total RNA that was extracted from the sputum cells as described elsewhere [18]. Quantitative RT-PCR (QRT-PCR) was carried out using the LightCycler SYBER Green PCR Master Mix (Roche LTD). The reaction mixture for HO-1 was initially heated to 95 °C for 10 min followed by 35 reaction cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s.

VEGF was initially heated to 95 °C for 10 min followed by 35 reaction cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. The following sense and antisense primers were used for QRT-PCR amplification: human HO-1 CCA GCG GGC CAG CAA CAA AGT GC -sense AAG CCT TCA GTG CCC AGC GTA AGG -antisense; human VEGF TGG GTG AGG TTT GAT CCG CAT AAT -sense AGG AGG AGG GCA GAA TCA TCA TCA CGA -antisense. Gene expression levels were normalized against the endogenous control GAPDH gene GTT GTC ATG GAT GAC CTT GGC -sense CCC ACC ATC ATC TTC CAG GAG -antisense.

The results are expressed as the ratio between the fold change in the target gene expression and the fold change in the reference gene expression (housekeeping gene GAPDH).

2.4. HO-1 protein activity

HO activity in the sputum supernatant was measured using a spectrophotometric assay of bilirubin production as described elsewhere [19]. Briefly, biliverdin reductase was recovered from a rat liver for the reaction. The reaction was conducted in the dark for 40 min at 37 °C and terminated by placing the samples on ice for 10 min. The amount of extracted bilirubin was calculated by the difference in absorption between 450-550 nm and an extinction coefficient of 40 mM⁻¹ cm⁻¹.

2.5. VEGF measurement by ELISA

VEGF protein content in the supernatant was evaluated by a commercial kit (R&D Systems, Inc.) which contains SF 21-expressed, recombinant human VEGF165 and antibodies raised against the recombinant protein.

2.6. Particle size distribution

Particle size analyses were performed according to a laser technique based on the time of transition theory using a CIS-1 Analyzer (Ankersmid, Israel). CIS-1 is a computerized inspection system for particle size analysis (PSA) in the range of 0.5–3600 μ employing laser measurement channel [20]. This system is based on principles of the laser obscuration/time of transition (TOT) theory.
A sample containing moving particles is scanned with a focused laser beam, rotating with a constant frequency by a wedge prism. Since the angular velocity is known, the size of each individual particle can be calculated from the duration and form of the beam obscuration signal. The TOT is directly related to the particle diameter: \( D = v \times t \), where: \( D \) = particle diameter, \( v \) = trajectory velocity of the laser beam, \( t \) = TOT. Three drops of sputum cell suspension were added to 3 ml of distilled water, and the particle measurement was performed long enough to reach 95% CI. The results of the particle size distribution are an average of three sequential measurements.

2.7. Statistical analyses

We performed all statistical analyses using the SPSS software version 13.0 for Windows. Demographic comparisons between groups were performed by parametric or nonparametric (Kruskal–Wallis) analysis of variance. Non-parametric data are presented as ranks. The Wilcoxon signed Ranks test, the Mann-Whitney test and the Chi-square test were used as appropriate. Significance was set to 0.05 correlation coefficient. Multiple comparisons between each pair were done using the Gabriel and Games-Howe tests. The association between the parameters was evaluated by Spearman correlation coefficients. Significance was set at \( P < 0.05 \).

3. Results

3.1. Pulmonary function testing and differential cell count

PFTs and lung volumes were normal for all participants. All 73 subjects underwent differential IS cell counts (Fig. 1). The Welder 2 group had significantly higher levels of neutrophils percentage and lower percentages of macrophages compared to both the Welder 1 group and the controls (\( 71 \pm 14% \) vs \( 42 \pm 28% \) and \( 47 \pm 21% \), \( P = 0.001 \) and \( 18 \pm 12% \) vs \( 42 \pm 26% \) and \( 38 \pm 20% \), respectively, \( P = 0.004 \)). The lymphocyte percentages were significantly lower in the Welder 2 group compared to both the Welder 1 group and the controls (\( 9.3 \pm 5.3% \) vs \( 15 \pm 8.7% \) and \( 14 \pm 7.1% \), respectively, \( P = 0.004 \)).

3.2. Particle size distribution

Particle size distribution was performed by laser particle scanner in samples recovered by IS. There were higher percentages of small particles (0–1 \( \mu \)m diameter) in W2 90.03 ± 0.78 vs 98.7 ± 2.81 in W1 and 93.4 ± 2.6 in C0 \( P < 0.04 \) and \( P < 0.02 \), respectively (Fig. 2a). For large particles (2–5 \( \mu \)m diameter) Welder 2 group display higher percentages compared to Welder 1 group and the controls. \( 2.4 \pm 1.3 \) vs \( 1.2 \pm 0.7 \) and \( 1.5 \pm 1 \) in \( 1 \pm 0.6 \) and \( 1.3 \pm 0.8 \), \( P = 0.04 \) (Fig. 2b).

3.3. VEGF and HO-1 protein levels and mRNA expression

Identification of VEGF and HO-1 gene expression in the sputum cells was done by RT-PCR assay (Table 2). The results revealed significantly high expressions of the HO-1 gene in the Welder 1 group and the controls (\( 151 \pm 134 \) and \( 223 \pm 165 \), respectively) compared to Welder 1 group (\( 190 \pm 141 \)).

![Fig. 1.](image1.png) *Fig. 1.* Differential cell counts - from induced samples of all participants \((n = 73)\) were performed by counting 300 cells in Giemsa-stained cytopreps. Gabriel test Multiple Comparison. 'Lymphocytes P < 0.041 (not significant adjusted to age) for Welders 1 vs Welders 2.' Neutrophils P < 0.008 for Controls vs Welders 2, P < 0.001 for Welders 1 vs Welders 2. 'Macrophages P < 0.002 for Controls vs Welders 2, P < 0.001 for Welders 1 vs Welders 2 vs Welders 1 (adjusted to age). The same data from part of Table 4 in our previous study [16].

![Fig. 2.](image2.png) *Fig. 2.* (a) Particles size distribution between 0 and 1 \( \mu \)m in all studied groups \((n = 73)\) was performed by laser particle scanner \((CIS-1), 0–1 \mu \)m diameter \( P < 0.02 \) for Controls vs Welders 2, \( P < 0.04 \) for Welders 1 vs Welder 2. \( P < 0.04 \) for Welders 1 vs Welder 2. Adjusted \( P \)-value \( = \) adjusted to age and smoking status. (b) Particles size distribution between 0 and 1 \( \mu \)m in all studied groups \((n = 73)\) \( \geq 5 \mu \)m diameter: \( P < 0.022 \) for Controls vs Welders 2, \( P < 0.04 \) for Welders 1 vs Welder 2, \( P < 0.0028 \) for Controls vs Welders 2, \( P < 0.004 \) for Welders 1 vs Welder 2 \( \geq 2 \mu \)m diameter. \( P \)-value \( = \) not adjusted; after adjustment the differences are not significant.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>HO gene in sputum</th>
<th>VEGF gene in sputum</th>
<th>HO protein activity in sputum (nM)</th>
<th>VEGF protein concentration in sputum (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welders 1</td>
<td>33.15 ± 14.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.95 ± 12.8</td>
<td>20.31 ± 15.91</td>
<td>151 ± 134</td>
</tr>
<tr>
<td>Welders 2</td>
<td>23.84 ± 10.41</td>
<td>36 ± 10.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.88 ± 12.25</td>
<td>223 ± 165</td>
</tr>
<tr>
<td>Controls</td>
<td>19.77 ± 13.56</td>
<td>18.88 ± 13.46</td>
<td>29.81 ± 12.11</td>
<td>190 ± 141</td>
</tr>
<tr>
<td>( P )-value*</td>
<td>0.02</td>
<td>0.004</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Welders 1, short-term exposure to aluminum/iron; Welders 2, long-term exposure to cadmium/chromium/iron/nickel. Adjusted \( P \)-value, adjusted to age and smoking status.

*Gabriel multiple comparison test was done to compare parameter between groups of Welders 1 vs Welders 2.

<sup>a</sup> HO-1 gene in sputum \( P < 0.02 \) Controls vs Welders 1.
which were low in the Welder 2 group and the controls (33.15 ± 14.64 vs 23.84 ± 10.41 and 19.77 ± 13.56, \( P = 0.02 \)). The VEGF gene expression was higher in the Welder 2 group than in the Welder 1 group and the controls (36 ± 17.42 vs 22.95 ± 12.8 and 18.88 ± 13.46, \( P = 0.004 \) (Table 2). The levels of protein of VEGF and HO-1 activity in the sputum supernatant were not significantly different between the groups.

### 3.4. Correlations between VEGF and HO-1 and particulate matter

Subject age and percentage of particles >2 μ and 2–5 μ correlated positively and significantly among all participants (\( r = -0.4 \) and \( r = 0.4, P = 0.001 \), respectively) (Table 3). VEGF concentration correlated significantly and positively with intracellular small particles, but there was a negative correlation for larger particles. Particles <2 μ and HO-1 protein activity also showed a highly positive correlation (\( r = 0.421 P = 0.006 \)). Neutrophil and VEGF protein concentrations correlated positively (\( r = 0.3 P = 0.045 \), as did HO-1 activity and macrophage percentages in sputum (\( r = 0.310 P = 0.032 \)). HO-1 activity was negatively associated with neutrophils (\( r = -0.298 P = 0.04 \)). Finally, HO-1 protein activity and HO-1 gene expression were positively correlated (\( r = 0.330 P = 0.05 \), Table 3).

### 4. Discussion

After having shown that asymptomatic welders who had been exposed to toxic fumes had evidence of occult inflammatory condition [16], we took this one step further and designed the current study to demonstrate the effect of the length of exposure of airborne particles associated with welding of metals on the molecular pathways of HO-1 and VEGF as representative markers of inflammatory and oxidative parameters among asymptomatic workers. To the best of our knowledge, our results are the first lines of evidence that the abnormalities in lung oxidative stress molecular pathways and biological mediators in apparently healthy workers dealing with welding processes are time dependant.

We had previously shown that IS can be used to study interstitial and occupational lung diseases [21]. In that context, particulate burden persists in the lungs for long periods after exposure and can be retrieved from IS samples. In our study on fire-fighters exposed to the hazardous dust mixture after the collapse of World Trade Center, we demonstrated the association between lung particle distribution and ambient particles in the exposed fire-fighters 10 months after the event [22].

In another earlier work [16], we reported that welders with long-term exposure to particles showed abnormalities in cell counts and an increase in oxidative stress markers in their exhaled breath condensates. In the present study, we expanded that investigation to include the involvement of the HO-1 enzyme during welding in correlation to VEGF and to exposure of particulate matter. We were able to demonstrate, for the first time, that the length of exposure over time was decisive in the differential expression of two genes, HO-1 and VEGF. Although we did not find any differences in proteins levels, we did demonstrate that the welders who had been exposed for longer periods of time had an increased expression of VEGF and a decreased expression of HO-1 in the mRNA recovered from their IS cells compared to welders who had been exposed for shorter periods of time.

The purpose of the present work was to study the involvement of the HO-1 enzyme in a priori recruited asymptomatic workers as related to particulate matter exposure over time. HO-1 is a mesenchymal enzyme that acts as an inducible defense against oxidative stress in models of inflammation hypoxia. Hyperoxia-mediated injury studies succeeded in measuring its activity in cellular extracts [23]. Unlike those works, we measured HO-1 activity enzyme in the extracellular fluid phase. Although HO-1 is an intracellular enzyme, we assumed that cells undergo apoptosis in the airways and that the cytoplasmic content is preserved in the lung lining fluid and reflects the HO-1 intracellular activity. We based our rationale on the fact that it is known that cells undergo apoptosis under inflammatory changes. It was shown that neutrophils undergo apoptosis due to inflammatory changes of airways induced by bacterial infection in cystic fibrosis patients [24]. Moreover apoptotic changes in the airways were also shown to be part of a physiologic maintenance of airways during endurance training of mice [25].

HO-1 plays a key role in the defense against oxidative stress, and its cytoprotective effects have already been demonstrated in the lungs [26] and other organs [27]. In our current study, high levels of HO-1 expression were revealed in the IS of only short-term exposed welders, while the HO-1 gene had apparently been silenced by the toxic effect of fuming—which is correlated with time—in welders exposed for longer periods of time. O’Harra et al. described a similar effect on airway epithelial cells after exposure to chromium [28].

On the other hand, VEGF gene expression was significantly higher in the group which had extended exposure to welding fume. High expression of VEGF apparently characterizes the lung’s response to extensive exposure of welding fumes. This extensive exposure induced a shifting of the stress condition resulting from high expression of the HO-1 gene to neutrophilic inflammation with high expression of the VEGF gene. Bussolati et al. [29] studied the association between HO-1 and VEGF in endothelial cell culture and found a controlling role for HO-1 in promoting angiogenesis. Pharmacologic inhibition of HO-1 induced marked leukocytic infiltration that enhanced VEGF-induced angiogenesis. Our results are in good agreement with theirs: we found low expression of HO-1 and high expression of VEGF with neutrophilia in the long-term exposed workers, while the short-term exposed group had high expression of HO-1 and low expression of VEGF without neutrophilia. It should be noted that several studies had shown that HO-1 can induce angiogenesis via secretion of VEGF [30,31], but none of those investigations were done in humans nor did they use a time-course dependant module.

The measurement of the intracellular particles by laser technology allowed a greater understanding of the correlation between particulate matter over time and HO-1 and VEGF protein secretion. We found that there is a preferential retention of the largest particles (2–5 μ) in the airways over the years of exposure, while the small particles (<2 μ) may be more easily translocated toward
the alveolar zone. We have already shown that there is a shift to ward fractions of small particles when samples are recovered from bronchoalveolar lavage compared to IS [18].

Our current results demonstrated a positive correlation between IS macrophages and HO-1 proteins secretion, with this HO-1 activity being associated with its gene expression. This suggests a possible mechanism by which the inhaled particles are internalized by macrophages, thereby promoting oxidative stress and high expression of the HO-1 gene. Similar results were found in vitro on cultured macrophages [32] and in vivo studies on animal models studies [33], but never before in humans.

The main role of macrophages is the uptake and clearance of inhaled microorganisms and environmental particles in the lung [34]. The inhalation of metal particles, however, induced macrophage apoptotic cell death [35]. This is compatible with our findings indicating a correlation between viable macrophages in sputum and the secretion of HO-1 in our set-up.

We further demonstrated that the intracellular presence of very small particles (<2μm) is well correlated to neutrophil influx and VEGF protein secretion in all welders, independent of length of exposure. Other studies also showed that neutrophils in sputum were found to be highly activated by inhaled particles [36].

It is well known that hydrogen peroxide induces VEGF secretion [37], and ultra-fine carbon black particles were shown to contribute to the increase of alveolar-capillary permeability and the induction of VEGF [38]. Induction of the VEGF gene does not induce secretion of the VEGF protein, indicating that there is a gap between the VEGF gene expression and its protein levels. Post-translational changes are known to characterize the movement transformation from gene to protein, especially in the case of VEGF [38]. Furthermore, the theory of VEGF compartmentalization must also be considered in this setting [39]. It was shown that proteins of a molecular mass like that of VEGF (i.e., 34–46 kDa) slowly diffuse across the alveolar epithelium. In this way, the high-level “reservoir” of VEGF protein in the respiratory epithelial surface plays a role in normal lung endothelial biology [40].

The main limitation of our work is the small number of welders in the group exposed for a long period of time. Those asymptomatic workers represent a homogeneous group that displays a clear-cut “healthy workers effect”. We expect that a study conducted on welders with clinical evidence of disability should demonstrate that the underlying inflammation had also induced impairment of pulmonary function.

In conclusion, this is the first analysis of the association between particle size distribution in IS and an inflammatory state in welders by means of cellular pattern and gene expression, with special emphasis upon the factor of time exposure. Our study results showed a significant influence of particle size distribution on inflammation and VEGF and HO-1 gene expressions in asymptomatic welders. These data are consistent with the view that inhaled particulate matter promotes airway abnormality. The presence of particles <2μm paralleled a positive correlation of HO-1 activity and macrophage percentage in IS cells. Taken together, these findings show that the lungs contend with moderate welding fume exposure by eliminating the stress condition through protective proteins, such as HO-1. Moreover, extended exposure to welding fumes promotes inflammation which causes changes in the angiogenic gene expression. Although these events occur after exposure to particulate matter, they were not seen in the functional parameters in our study. Prospective studies using molecular parameters combined with close clinical monitoring of disease are warranted to understand the mechanisms of welding-related disease development and to assess whether the noninvasive technique of IS may be used for biological monitoring of exposed workers.


