The role of Fe(II) in Dust Induced Carcinogenesis

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16. Abstract (Limit: 200 words) The carcinogenicity of ferrous ion in solution and ferrous containing particles was investigated in Syrian-hamster-embryo (SHE) and human-tracheal-epithelial cells in-vitro. An attempt was made to determine the mechanism of ferrous containing dust induced carcinogenesis and to compare the responsiveness of oxidative stress response transcription factors to ferrous ion treatments. The mechanism of ferrous coated dust induced carcinogenesis was investigated using dichorofluorescein (DCF) and 8-oxo-deoxyguanosine formation. Findings indicated that ferrous ion in aqueous solution is cytotoxic, but not transforming in SHE cells. In contrast, when ferrous ion was deposited on a particle and then phagocytized by these cells, the released ferrous ion gave rise to cellular transformation. These findings suggested that ferrous ions in solution were difficult to incorporate into cells, and when internalized, any free iron would be strongly associated with proteins such as ferritin and transferrin and therefore not be available. Since the redox activity of ferrous ion is pH dependent, a low pH in the stomach can kinetically render the ion inactive. Once a particle containing ferrous ion is inhaled, the ferrous ion will be subsequently converted in the lung to ferric ion, resulting in the formation of reactive oxygen species, which can damage lung cells and cause cytotoxicity.

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Increasing evidence demonstrates that an excess of lung cancer and stomach cancer has been observed in iron ore and steel factories workers. It has been shown that the incidence of cancer is related to exposure to mineral dusts. Ferrous iron (Fe^{2+}) is capable of producing reactive oxygen species (ROS) such as $OH^{\bullet}$ and ferryl species ($Fe^{4+}=O$) and may play an important role in dust-induced carcinogenesis and lung injury. The original specific aims of this project were:

1) To compare the carcinogenicity of Fe^{2+} ion in solution and Fe^{2+}-containing dusts. We hypothesized that phagocytosis of Fe^{2+}-containing dusts may increase the ambient concentration of ROS in cells which can lead to DNA damage and finally cell transformation.

2) To determine the mechanism of Fe^{2+}-containing dust-induced carcinogenesis. Dichlorofluorescein (DCF) assays to estimate oxidants level in cells, and 8-oxo-deoxyguanosine (8-oxo-dG) formation, would be quantitated on both Syrian hamster embryo (SHE) cells and human tracheal epithelial (HTE) cells following exposure to different iron compounds.

3) To compare the responsiveness of oxidative stress-responsive transcription factors such as nuclear factor κB (NF-κB) and AP-1 to Fe^{2+} treatments.

Significant Findings

Specific Aim #1:

The carcinogenicity of Fe^{2+} ion in solution and Fe^{2+}-containing particles was investigated in an *in vitro* system. $FeSO_4$ in solution, freshly prepared water insoluble iron sulfite (FeS), and water insoluble hydrated iron oxide (FeOOH) were tested in SHE cell transformation assays. We found that hydrated iron oxide (Fe^{3+}, in forms of FeOOH) and $FeSO_4$ in solution were not able to transform SHE cells (Table 1). Chemically, Fe^{3+} is a weak oxidant and can only be activated by reducing agent such as $O_2^{-\bullet}$ to Fe^{2+}, which is probably the active component in transforming SHE cells when incorporated in the cells. Fe^{2+} in solution was difficult to get into cells to produce ROS that attack DNA in the nuclei. This was likely due to the alkaline pH of the cell growth medium. It is known that oxidation of Fe^{2+} to Fe^{3+} resulting in ROS formation is greatly dependent upon the pH of the media (i.e. $t_{1/2}$ at pH 7: 8 min, but 3 years at pH 3.5). Therefore, the short-lived ROS produced by $FeSO_4$ in the excellular medium damaged

the cell membrane which caused cell toxicity, but no transformation was observed. Hydrated iron oxide (FeOOH) did not cause cytotoxicity, and the transformation frequency after treatment was not significantly different from the spontaneous transformation frequency of the control SHE cells. In contrast, FeS transformed SHE cells and caused toxicity in SHE cells at higher doses. This was due to the pinocytosis of the Fe^{2+} containing particles by SHE cells and the released Fe^{2+} in the cells induced transformation.

Specific Aim #2:

The mechanisms of Fe^{2+}-coated dust-induced carcinogenesis were investigated by dichlorofluorescein (DCF) and 8-oxo-deoxyguanosine (8-oxo-dG) formation. Two sets of FeS were prepared in order to have different amount of $FeSO_4$ coated on the particles. The freshly ground FeS was divided and placed in the upper part of two dessicators containing drierite or saturated aqueous solution of $MnCl_2 \cdot 4H_2O$ and left to age (to be oxidized in the presence of oxygen and humidity to form a coating of $FeSO_4$). The saturated solution was allowed to establish a relative dessicator humidity of 57.6%. The FeS aged under this experimental condition was designated as "wet FeS" and the FeS aged under direrite was called "dry FeS". After nine months of aging, we found that dry FeS contained 0.85% (w/w) of water soluble Fe^{2+} and the wet FeS contained 3.38% of Fe^{2+} as detected by 2,2'-bipyridine.

Since iron is a first raw transition element that can exist in several oxidation states, oxidative stress may be one of the most important mechanisms in iron-induced cell transformation. The intracellular concentration of oxidants can be directly measured by dichlorofluorescin diacetate. An increase in DCF fluorescence intensity indicates that strong oxidants such as H_2O_2 and lipid hydroperoxides are formed within the cells after treatment. We found that $FeSO_4$, dry FeS, wet FeS, and hydrated iron oxide increased the formation of DCF in SHE cells (Table 2). Among the four iron compounds tested, $FeSO_4$ had the most prononced effect on DCF formation. Table 3 shows that wet FeS, dry FeS, and FeSO4 significantly increased the formation of DCF in HTE cells. In contrast, hydrated iron oxide which contains only Fe^{3+} did not have any significant enhancing effects on the DCF formation. These results suggest that the overall increase in oxidants formation as detected by DCF may be due to stress induced by the Fe^{2+} particles, but can not explain why only Fe^{2+} compounds induced cell transformation.

8-oxo-dG is an important DNA adduct which can cause α-polymerase to miscode incoporation of nucleotides in the replicated strands. We found that, in a cell free system, dry FeS, $FeSO_4$, wet FeS induced large amounts of 8-oxo-dG in calf thymus DNA after 2 hrs of incubation. In contrast, FeOOH had no significant effect on the formation of 8-oxo-dG (Table 4). When human tracheal epithelial (HTE) cells were treated with the above-cited iron compounds, we found that Fe^{2+} containing particles such as FeS was able to induce 8-oxo-dG formation after 8 hrs treatment. In contrast, the level of 8-oxo-dG in HTE cells treated with FeOOH was not detectable (Table 5). These results suggest that Fe^{2+} can oxidize DNA and my be responsible for the observed cell transformation.

Specific Aim #3:

Above-normal levels of ROS produced by excessive Fe^{2+} exposure may induce oxidative stress. Activator protein-1 (AP-1) is an inducible mammalian transcription factor that can be activated when cells are exposed to various growth factors, tumor promoters, and ROS. We found that FeS induced an overexpression of AP-1 in HTE cells after 5 hrs treatment, indicating that Fe^{2+} is an active inducer of H_2O_2 and $O_2^{-\bullet}$.

Other Findings:

Our *in vitro* studies have shown that Fe^{2+} is an active component in damaging cells when incorporated within cells. Due to the high rate of redox activity of Fe^{2+} at pH>7, we believe that Fe^{2+} may be a potential lung hazard because of the neutral pH of lung medium. We hypothesize that Fe^{2+} may be the active compound in inducing coal workers' pneumoconiosis (CWP). There are strong indications that the ability of alveolar macrophages (AMs) to dissolve inorganic particles is due to the low pH in the phagolysosomes. One of the physico-chemical parameters that can influence the dissolution of a particle is its buffering capacity, which can be defined as the ability of coals to maintain the pH of aqueous coal suspension. Fe^{2+} in low buffering capacity coal would be dissolved in the phagolysosomes of macrophages following phagocytosis, and then induce oxidative stress which lead to lung injury and CWP development. In contrast, high buffering capacity coal may prevent acid solubilization of Fe^{2+} and thus render the coal less hazardous. To validate this hypothesis, coal samples from Pennsylvania and Utah coal mines were purchased from the Penn State Coal Sample Bank. Table 6 shows that the coals from central Pennsylvania on average released 2500 ppm of Fe^{2+} at pH 4.5. These coals were acidic and thus had little buffering capacity.

Epidemiological studies have shown that CWP is frequent (26%) in the coal workers of central Pennsylvania. In contrast, coals from Utah were neutral or basic in aqueous medium. On the average, these coals needed 469.2 µl of 1 M H_2SO_4 to reduce the pH of aqueous coal suspensions to 4.5 and had little acid soluble Fe^{2+} (0.3 ppm). CWP prevalence of 4% was reported in the coal workers of Utah. The coals from southwestern West Virginia (10% CWP) had low buffering capacity but a moderate level of acid soluble Fe^{2+} (11 ppm). These results suggest that Fe^{2+} may be responsible for the lung injury induced by coal dusts.

Figure 1 shows the difference between the two coal samples from the Utah and Pennsylvania coal mines in induction of AP-1 binding to consensus target DNA in HTE cells (2nd band on the gel). The coal from the Pennsylvania coal mine with a high acid soluble Fe^{2+} activated AP-1 to the same extent as 10 µM H_2O_2. In contrast, the coal from the Utah coal mine without acid soluble Fe^{2+} had no effect on the activation of AP-1. The uppermost band on the gel showed the same intensity of the non-specific binding in the lanes B, C, D, and E, indicating that the loading of the nuclear proteins was the same among the four samples. The lowest band originated from the free excess AP-1 probe. These results indicate that the coal from Pennsylvania can upregulate the expression of AP-1 in order to protect cells from oxidant attack.

Usefulness of Findings:

Unlike chromium, nickel, and arsenic, which have been found to be carcinogenic and lung hazardous in humans and experimental animals, the carcinogenicity of iron in mammals is still under debate and its adverse effect on the lung is not well known. The carcinogenicity of metallic iron and iron oxide (Fe_2O_3, ferric iron, Fe^{3+}) in experimental animals was negative, with mostly benign non-metastisizing tumors appearing. However, the carcinogenicity of ferrous iron compounds has not been adequately tested in cells and not tested at all in experimental animals.

Findings from this study have demonstrated that Fe^{2+} in aqueous solution is cytotoxic, but not transforming in SHE cells. In contrast, when Fe^{2+} is deposited on a particle (i.e. FeS), and then phagocytized by these cells, the released Fe^{2+} gave rise to cellular transformation. Thses results suggest that Fe^{2+} ions in solution were difficult to incorporate into cells and, when internalized, any free iron would be strongly associated with proteins such as ferritin and transferrin and therefore not available. Phagocytosis of a particle containing Fe^{2+} delivered a large quantity of free Fe^{2+} which was then able

to produce ROS as shown by DCF formation. The formed ROS may attack DNA by inducing 8-oxo-dG formation and AP-1 overexpression, and ultimately giving rise to transformed cells.

Since the redox activity of Fe^{2+} is pH dependent, a low pH in the stomach (pH 1-2) can kinetically render the Fe^{2+} inactive (reaction half life of 3 years), such that transferrin and ferritin can be regulated and synthesized, and thus trap the Fe^{2+} in the mucosa. In therapeutics, iron deficiency is treated orally with a variety of ferrous salts and certain iron carbohydrate complexes are given in parenteral therapy. On the other hand, our studies strongly suggest that inhalation of Fe^{2+}-containing particles may induce pulmonary injury. In lung medium in which the pH is usually greater than 7, inhalation of Fe^{2+}-containing dusts will result in rapid oxidation of Fe^{2+} to Fe^{3+} and ROS formation. We have shown in the present study that the prevalence of coal workers' pneumoconiosis in US coal mines associates positively with the content of Fe^{2+} in the coal dusts. Fe^{2+}-containing particles were capable of transforming SHE cells and inducing cell cytotoxicity in HTE cells.

The component of air particulate matter is a heterogeneous mixture that varies in constituent particle sizes and chemical composition. Iron is a prominent element on earth and throughout the solar system. Among all elements on the surface of the Earth, it ranks fourth in abundance and is second only to aluminum among the metals. Thus, there is ample opportunity for exposure to iron both in and outside the work place. Sulfur dioxide (SO_2) and particulate matter are generated together by fossil fuel combustion. In the presence of humidity, SO_2 can form H_2SO_4 on the surface of particles which solubilzes iron oxide (FeO) and siderite ($FeCO_3$) yielding $FeSO_4$. Moreover, H_2SO_4 can lower the pH and thus stabilize the $FeSO_4$ formed on the particles surface. Once a particle containing $FeSO_4$ is inhaled, Fe^{2+} will be subsequently converted in the lung to Fe^{3+} resulting in ROS, which can damage lung cells and cause cytotoxicity. Therefore, while iron deficiency anemia should be diagnosed, treated and if possible prevented, there good reasons to avoid unecessary exposure to Fe^{2+} containing dusts. The role of reactive Fe^{2+} in dusts is a parameter which is worth investigating in order to predict which dusts will lead to a higher incidence of lung injury.

Relation of Publications to Project Aims

Xi Huang, Henri Pezerat, Zoe Elias, and Max Costa (1997) **The role of Fe(II) in dust-induced carcinogenesis.** This publication is related to Specific Aims 1, 2, and 3. The results of SHE cell transformation, DCF and 8-oxo-dG assays in both HTE cells and SHE cells, and AP-1 expression will be presented. This paper emphasizes the physical forms of iron compounds (i.e. ferrous or ferric, water soluble or water insoluble) in induction of cell transformation and cytotoxicity. These results also suggest that the adverse effect of Fe^{2+} may be organ specific. The lung may be the primary target.

Xi Huang, Roger Zalma, Max Costa, and Henri Pezerat (1997) **Oxidant-producing activity of ferrous sulfate in aqueous solution.** This publication is related to the Specific Aim 2. It presents the results of our physico-chemical studies in elucidating the mechanisms of Fe^{2+} to induce ROS. We found that factors such as the concentration of Fe^{2+}, ratio Fe^{2+}/Fe^{3+}, the concentration and the pH of the media, the presence of O_2 in the media played important roles in the kinetics and yield of Fe^{2+}-induced ROS formation.

Xi Huang, Jeanine Fournier, Karen Koenig, and LungChi Chen (1997) **Acid soluble Fe(II) and buffering capacity of coal: Role in coal workers' pneumoconiosis.** This publication is related to Specific Aims 1 and 2, though the work was not originally proposed in this project. The results in this paper strongly support our hypothesis that Fe^{2+} may play an important role in dust-induced lung injury and carcinogenesis.

Table 1. Cytotoxicity and rate of SHE transformation induced by $FeSO_4$ solutions, goethite (FeOOH) and ferrous sulfite (FeS) particles[a]

Iron compounds	Dose	Cytotoxicity (%)[b]	Transformation (%)[c]
FeOOH ($\mu g/cm^2$)	0	-	0.14
	1.9	0	0.11
	3.8	0	0.12
	7.6	0	0.21
	15.2	0	0.10
$FeSO_4$ (mM)	0	-	0.25
	0.1	5.1	0.36
	0.5	40.2	0.5
	1.0	78.3	0
FeS ($\mu g/cm^2$)	0	-	0.12
	0.95	0	0.80
	1.9	0	1.15
	3.8	15.5	2.32
	5.7	63.7	4.93
	7.6	82.5	6.44

a. The iron compounds were suspended in sterile tri-distilled water immediately before cell treatment. The concentrations tested ranged from the non-toxic to 85% cytotoxic concentrations, established by preliminary cloning efficiency (CE) assays on SHE cells.
b. The cytotoxicity was determined by using the relative CE, i.e. the CE of the treated cells divided by the CE of the control cells x 100.
c. The frequency of morphological transformation was calculated as the number of transformed colonies divided by the total number of surviving colonies, multiplied by 100. The data presented the combined results of two separate experiments, and the total number of colonies was around 1000 per dose (10 to 12 dishes were used per dose).

Table 2. Formation of DCF in intact SHE cells induced by different iron compounds[a]

Dose	Fluorescence Intensity (% of control)			
($\mu g/cm^2$)	Dry FeS	Wet FeS	FeOOH	$FeSO_4$
5	138.6 ± 1.6*[b]	120.1 ± 2.1	121.4 ± 11.1	228.9 ± 13.5*
10	173.9 ± 6.2*	176.3 ± 12.8*	153.7 ± 10.3*	304.9 ± 13.2*
15	153.7 ± 14.9*	154.5 ± 3.5*	146.4 ± 2.8*	366.8 ± 31.1*
20	120.2 ± 10.9	199.5 ± 2.6*	129.1 ± 7.5	583.0 ± 80.0*

a. SHE cells were treated with these iron compounds for 5 hrs and followed by DCF-dAc (50 μM) for 1 hr. Controls received everything except iron. The data were obtained from three independent experiments and expressed as the percent of control ± SE.

b. Significantly different from control, $p < 0.05$.

Table 3. Formation of DCF in intact HTE cells induced by different iron compounds[a]

Dose	Fluorescence Intensity (% of control)			
($\mu g/cm^2$)	Wet FeS	Dry FeS	FeOOH	$FeSO_4$
5	177.2 ± 1.8*[b]	137.8 ± 2.6	83.5 ± 2.2*	123.7 + 16.7
10	175.8 ± 6.2*	87.9 ± 23.6	82.8 ± 5.0*	140.5 ± 3.8
15	270.6 ± 5.1*	144.2 ± 20.3	100.2 ± 4.9	152.7 ± 10.8*
20	247.8 ± 21.3*	165.3 ± 5.4*	88.7 ± 1.5	167.6 ± 11.5*

a. HTE cells were treated with these iron compounds for 5 hrs and followed by DCF-dAc (100 μM) for 1 hr. Controls received everything except iron. The data were obtained from three independent experiments and expressed as the percent of control ± SE.

b. Significantly different from control, $p < 0.05$.

Table 4. 8-oxo-dG formation in calf thymus DNA induced by different iron compounds

Compound	Dose (mg/ml)	8-oxo-dG/10^5 dG[a]
Blank	-	1.5
FeS	10	163.8
Aged FeS	10	289.7
FeOOH	10	7.3
$FeSO_4$	2	240.9

a. 500 μg of calf thymus DNA were incubated with different iron compounds in 1 ml of PBS (10 mM, pH 7.0). The mixtures were shaken for 2 hrs at room temperautre, and then centrifuged to remove iron particles. The treated DNA samples were precipitated by alcohol and digested by nulease p1, alkaline phosphatase, and acid phosphatase. The digested DNA were analyzed on HPLC coupled with UV and electro-chemical (EC) detectors.

Table 5. 8-oxo-dG formation in human tracheal epithelial cells after treatment with iron compounds

Compound	8-oxo-dG/10^5 dG
Control	
8 hrs	ND[a]
24 hrs	ND
Dry FeS (5 $\mu g/cm^2$)	
8 hrs	2.96
24 hrs	3.54
FeOOH ($\mu g/cm^2$)	
8 hrs	ND
24 hrs	ND

a. ND: not detectable

Table 6. pHs, buffering capacities, and acid soluble Fe^{2+} contents of thirty coal samples from three coal mine regions with different prevalence of CWP

PSOC n°	Seams	County	m/yr	Sample type	pH	Buffering capacity (µl)	Fe^{2+} (ppm) at pH 4.5
UT (4% CWP)							
313	Hiawatha	Carbon	7/74	Chan-Seam	7.98	150	0[a]
498	Hiawatha	Emery	8/76	Chan-Sect	6.16	240	0
500	Hiawatha	Emery	8/76	Chan-Sect	8.70	65	0
1502	Hiawatha	Sevier	9/85	Chan-Work	8.76	505	0
433	Upper Hiawatha	Servier	6/76	Chan-Sect	7.55	342	0
432	Upper Hiawatha	Servier	6/76	Chan-Sect	7.50	1455	3
431	Upper Hiawatha	Servier	6/76	Chan-Work	7.39	985	30
429	Upper Hiawatha	Servier	6/76	Chan-Seam	7.12	505	0
459	Lower Sunnyside	Emery	6/76	Chan-Seam	7.37	160	0
1112	Blind Canyon	Emery	8/78	Chan-Seam	6.38	285	0.4
						469.2 ± 412.78 (SD)	0.33 ± 0.89
WV (10% CWP)							
827	Sewell	Nicholas	7/77	Chan-Sect	6.65	5	0
826	Sewell	Nicholas	7/77	Chan-Sect	4.48	0	0
825	Sewell	Nicholas	7/77	Chan-Sect	4.18	0	51
824	Sewell	Nicholas	7/77	Chan-Seam	5.32	2	12
823	Sewell	Nicholas	7/77	Chan-Seam	6.58	5	0.9
822	Sewell	Nicholas	7/77	Chan-Seam	5.69	2	3
731	Sewell B	Randolph	6/77	Chan-Sect	6.05	2	4
730	Sewell B	Randolph	6/77	Chan-Sect	4.69	2	20
729	Sewell B	Randolph	6/77	Chan-Sect	6.97	2	4
728	Sewell B	Randolph	6/77	Chan-Seam	6.21	5	0.4
727	Sewell B	Randolph	6/77	Chan-Seam	5.48	3	60
726	Sewell B	Randolph	6/77	Chan-Seam	4.23	0	25
						2.33 ± 1.87	11 ± 15

PA (26% CWP)							
260	Middle Kittanning	Clearfield	3/73	Chan-Seam	4.54	2	509
324	Middle Kittanning	Clearfield	9/74	Chan-Seam	3.33	0	2546
325	Middle Kittanning	Armstrong	10/74	Chan-Seam	1.95	0	5245
337	Lower Kittanning	Jefferson	4/75	Chan-Seam	3.15	0	1677
1197	Lower Kittanning	Somerset	11/79	Chan-Seam	3.56	0	1477
1198	Lower Kittanning	Indiana	11/79	Chan-Seam	2.67	0	6877
1313	Lower Kittanning	Clearfield	6/80	Chan-Seam	2.47	0	806
1516	Lower Kittanning	Somerset	5/86	Chan-Seam	3.30	0	1202
						0.25 ± 0.71	2500 ± 2300

PSOC n°: sample designation number from the Penn State Coal Sample Bank; m/yr: month/year the coal was sampled; Chan-Seam: full seam channel samples; Chan-Work: channel samples limited to the vertical extent of the seam being mined; Chan-Sect: channel samples of seam subsection; pH: pH of suspensions of coal samples in distilled water (2.7 g in 30 ml of H_2O); Buffering capacity: microliters (μl) of 1 M H_2SO_4 needed to reduce the pH of the above aqueous coal suspension to pH 4.5; Acid soluble Fe^{2+}: ppm of coal released in 10 mM phosphate solution pH 4.5; a: not detectable.

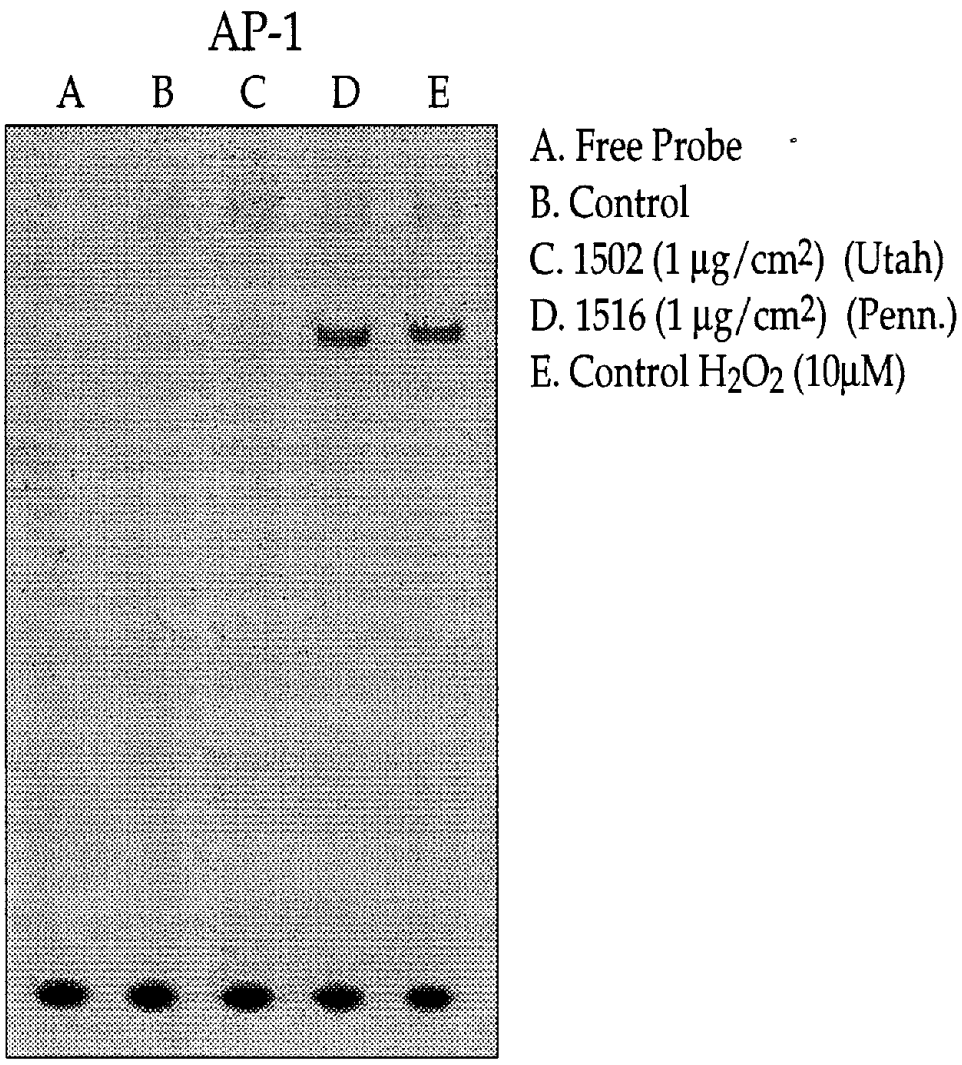
AP-1
A B C D E
A. Free Probe
B. Control
C. 1502 (1 µg/cm2) (Utah)
D. 1516 (1 µg/cm2) (Penn.)
E. Control H2O2 (10µM)

Figure 1