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Original Article

Identification of Intracellular Sources Responsible for Endogenous Reactive Oxygen Species Formation

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Abstract

The endogenous reactive oxygen species ("ROS") formation is associated with many pathologic states such as inflammatory diseases, neurodegenerative diseases, brain and heart ischemic injuries, cancer, and aging. The purpose of this study was to investigate the endogenous sources for "ROS" formation in intact isolated rat hepatocytes, in particular, peroxisomal oxidases, monoamine oxidase, xanthine oxidase, cytochrome P450, and mitochondria electron transport. The rat hepatocyte catalyzed oxidation of 2',7'-dichlorofluorescin to form the fluorescent 2,7'-dichlorofluorescein was used to measure endogenous and xenobiotic-induced reactive oxygen species ("ROS") formation by intact isolated rat hepatocytes. Various oxidase substrates and inhibitors were then used to identify the intracellular oxidases responsible. Endogenous "ROS" formation was markedly increased in catalase inhibited or GSH depleted hepatocytes, and was inhibited by "ROS" scavengers or desferoxamine. Endogenous "ROS" formation was also inhibited by cytochrome P450 inhibitors, but was not affected by oxypurinol, a xanthine oxidase inhibitor. Mitochondrial respiratory chain inhibitors or hypoxia, on the other hand, markedly increased "ROS" before cytotoxicity ensued. This suggests endogenous "ROS" formation can largely be attributed to oxygen reduction by reduced mitochondrial electron transport components and reduced cytochrome P450 isozymes. Addition of monoamine oxidase substrates increased antimycin A-resistant respiration and "ROS" formation before cytotoxicity ensued. On the other hand peroxisomal substrates readily induced "ROS" formation and were cytotoxic towards catalase inhibited hepatocytes, which suggests that peroxisomal catalase removes endogenous H_2O_2 formed in the peroxisomes. The consequences of upregulation of peroxisomal oxidases are discussed.

Keywords: Reactive oxygen species; Peroxisomes; Monoamine oxidase; Catalase; Mitochondria; Dichlorofluorescin; Hepatocyte.

Introduction

The cellular generation of reactive oxygen species ("ROS") has been associated with, and may contribute to human disease states such as inflammatory diseases, neurodegenerative diseases, ischemia-reperfusion injury, cancer, and aging (1). It has also been associated with tissue reoxygenation following hypoxia or anoxia (2), and cytotoxicity induced by endobiotics and xenobiotics (3-5).

Cellular sources of "ROS" production include plasma membrane NADPH oxidase and intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases and mitochondrial electron transport components. The latter is considered to be the major source of "ROS" that has been implicated in a number of diseases and disorders (6). However, this is mostly based on experiments using submitochondrial particles obtained by

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sonication (7). Recently, hydrogen peroxide (H_2O_2) formation by intact mitochondria isolated from monocytes/macrophages was measured with luminol as a chemlumigenic probe and shown to be markedly increased by antimycin A (AA), an inhibitor of ubiquinol-cytochrome c reductase (8).

Recently, "ROS" formation in intact cells has been measured by loading cells with 2',7'-dichlorofluorescin diacetate, which diffuses through the cell membrane and is trapped in the cell by being incorporated into cellular lipids and enzymatically deacetylated by intracellular esterases to form 2',7'-dichlorofluorescin (DCFH). The latter is rapidly oxidized to form the highly fluorescent 2',7'-dichlorofluorescein (9) by intracellular "ROS" (e.g., hydroxyl radicals, ferryl species) as H_2O_2 or superoxide anion (O_2^-) cannot directly oxidize DCFH (1). This method has recently been used to show that cellular "ROS" formation is induced by cyanide (10), aflatoxin B1 (4), capsaicin (11), ethanol (5), and taurochenodeoxycholic acid (12).

Recently, this method has been used to show that "ROS" formation by isolated intact hepatocytes was markedly increased by nitrofurantoin, an antimicrobial agent. This was attributed to redox cycling of the nitro group and its radical anion which reductively activates oxygen (13). Various metals also induced hepatocyte "ROS" formation before cytotoxicity ensued. The comparative effectiveness of metals (at a cytotoxic dose) for inducing "ROS" formation was $CuCl_2$ > $K_2Cr_2O_7$ > $HgCl_2$ > $CdCl_2$ (3, 14, 15). Furthermore the cytotoxicity induced by these metals was prevented by the hydroxyl radical scavengers dimethyl sulfoxide or mannitol (3, 14, 15). It was also demonstrated that lysosomal lipid peroxidation preceded $CuCl_2$ or $K_2Cr_2O_7$ induced hepatocyte cytotoxicity (16).

In this study the endogenous sources for "ROS" formation in intact isolated rat hepatocytes, in particular, peroxisomal oxidases, monoamine oxidase, xanthine oxidase, cytochrome P450, and mitochondria electron transport has been investigated.

Experimental

Chemicals

1-Bromoheptane, and N,N'-diphenyl-1,4-phenylenediamine (DPPD) were obtained from Aldrich Chemical Co. (Oakville, ON, Canada). Collagenase (from Clostridium histolyicum), bovine serum albumin (BSA) and HEPES were purchased from Boehringer-Mannheim (Montreal, Quebec, Canada). Trypan blue, d-mannitol, dimethyl sulfoxide, catalase, superoxide dismutase, chloroquine diphosphate, methylamine HCl, 3-methyl adenine, monensin sodium, thiobarbituric acid, ethylene glycol-bis (p-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA), sodium pentobarbital and heparin were obtained from Sigma (Oakville, ON, Canada). 2',7'-Dichlorofluorescin diacetate was purchased from Fluka Chemie AG (Oakville, ON, Canada). Desferoxamine was a gift from Ciba-Geigy Canada Ltd. (Toronto, ON, Canada). All chemicals were of the highest commercial grade available.

Animals

Male Sprague-Dawley rats (280-300g), fed a standard chow diet and given water *ad libitum* were used in all experiments.

Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver as described by Pourahmad and O'Brien, (2000). Approximately 85-90% of the hepatocytes excluded trypan blue upon isolation (i.e., 85-90% cell viability). Cells were suspended at a density of 106 cells/ml in round bottom flasks, rotating in a water bath maintained at 37°C in Krebs-Hensleit buffer (pH 7.4), supplemented with 12.5 mM HEPES under an atmosphere of 10% O_2 : 85% N_2 : 5% CO_2. Glutathione (GSH) depleted hepatocytes were prepared by preincubation of hepatocytes with 200 µM 1-bromoheptane for 30 minutes as described by Khan and O'Brien, (1991). Catalase inactivated hepatocytes were prepared by preincubation of hepatocytes with 4mM azide for 5 min.

Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion assay. Aliquots of the hepatocyte incubate were taken at different time points during the 3 hours incubation period. At least 80-90% of the control cells were still viable after 3h.

Determination of "ROS"

To determine the amount of hepatocyte "ROS" generation, 2',7'-dichlorofluorescin diacetate was used as it penetrates the cells and becomes hydrolyzed by an intracellular esterase to form DCFH. The latter reacts with intracellular "ROS" to form the highly fluorescent 2',7'-dichlorofluorescein, which effluxes the cell. Hepatocytes (10^6 cells/ml) were suspended in 10ml modified Hank's balanced salt solution, adjusted to pH 7.4 with

Table 1. Endogenous "ROS" formation by isolated hepatocytes

Additions	Incubation time:	DCFH Oxidation (fluor. units) 30 min	60 min	120 min
Control hepatocytes		68±6	77±7	88±7
+ Catalase inhibitors				
cyanamide (0.2mM)		118±10*	120±11*	145±11*
azide (4mM)		89±8*	95±8*	108±9*
+ MAO/Xanthine oxidase inhibitors				
phenelzine (0.01mM)		61±6	72±8	91±10
oxypurinol (0.05mM)		66±7	74±8	86±13
+ "ROS" scavengers				
dimethylthiourea (5mM)		41±4*	46±5*	58±5*
dimethylsulfoxide (125mM)		54±5*	49±5*	55±6*
quercetin (0.02mM)		54±5*	49±5*	55±6*
desferoxamine (0.5mM)		59±6	64±7	68±6*
+ P450/P450 reductase inhibitors				
diphenylene iodonium chloride (0.05mM)		39±5*	41±3*	41±6*
1-benzylimidazole (0.1mM)		43±4*	43±5*	41±5*
cimetidine (1mM)		41±4*	53±6*	58±4*
SKF525A (0.05mM)		43±3*	62±5*	78±8*
+ P450 activator				
H_2O_2 (glucose/glucose oxidase)[a]		153±12	204±19	135±12
+ 1-benzylimidazole (0.1mM)		57±6**	67±6**	72±7**
+ cimetidine (1mM)		61±5**	61±7**	67±8**
GSH depleted hepatocytes		88±9*	98±11*	113±13*

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Hensleit buffer, pH 7.4 at 37°C under 10% O_2, 5% CO_2, 85% N_2. DCFH oxidation to fluorescent dichlorofluorescein was used to determine "ROS" formation as described in Materials and Methods. Values are expressed as the means of three separate experiments (±SD), where the accepted level of significance was $p < 0.05$.

[a] H_2O_2 was generated with 15mM glucose and 1.5 U/ml glucose oxidase.

* Significant difference from control hepatocytes

** Significant difference from P450 activated hepatocytes.

Table 2. Hepatocyte "ROS" modulation by mitochondrial toxins.

Additions	DCFH oxidation (fluor. units)			%Cytotoxicity
Incubation time:	30 min	60 min	120 min	120 min
Control hepatocytes	74±7	88±12	106±12	17±3
+ rotenone (0.02mM)	180±20*	290±25*	284±40*	33±4*
+ AA (0.01mM)	205±29*	282±43*	349±62*	55±6*
+ cyanide (1mM)	123±13*	179±20*	251±26*	38±4*
+ hypoxia (90 min)	174±18*	245±22*	283±51*	54±6*
+ CCCP (0.002mM)	54±6**	64±5**	74±8**	34±3*
+ pentachlorophenol (0.01mM)	64±6**	68±7**	76±7**	24±4

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Hensleit buffer, pH 7.4 at 37°C under 10% O_2, 5% CO_2, 85% N_2. DCFH oxidation to fluorescent dichlorofluorescein was used to determine "ROS" formation as described in Materials and Methods. Cytotoxicity was determined as the percentage of cells taking up trypan blue. Values are expressed as the means of three separate experiments (±SD), where the accepted level of significance was $p < 0.05$.

* Significantly greater than control hepatocytes

** Significantly less than control hepatocytes

10mM HEPES and were incubated with various test compounds for 30, 60, and 120 minutes. After centrifugation (50 x g, 1 min) the cells were resuspended in Hank's balanced salt solution (adjusted to pH 7.4 with 50mM Tris-HCl) and loaded with DCFH by incubating with 2',7'-dichlorofluorescin diacetate for 2 min at 37°C. The fluorescence intensity of the 2',7'-dichlorofluorescein formed was monitored at 500nm (emission) and at 520nm (excitation). The results were expressed as fluorescent intesity per 106 cells (4).

Cyanide-resistant respiration

The oxygen concentration of 2 ml aliquots of the hepatocyte suspension (10^6 cells/ml in Krebs-Hensleit buffer, pH 7.4) were recorded continuously using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Ohio, USA) after the addition of 1mM cyanide to inhibit mitochondrial respiration. After 4 min, various oxidase substrates were added and the rate of cyanide-resistant respiration was measured so as to estimate the rate of H_2O_2 formation by the intracellular oxidases.

Statistical analysis

The statistical significance of differences between the control and treatment groups in these studies was determined by *t*-test. Results represent the standard deviation (±SD) of triplicate samples. The minimal level of significance chosen was $p < 0.05$.

Results and Discussion

As shown in Table 1, DCFH oxidation by control hepatocytes increased steadily with time and was higher in catalase inactivated or GSH depleted hepatocytes. Further evidence that DCFH oxidation was a measure of hepatocyte endogenous "ROS" formation was the inhibition of DCFH oxidation by quercetin (ROS scavenger), dimethylthiourea or dimethylsulfoxide (hydroxyl radical scavengers), or desferoxamine (ferric ion chelator). Endogenous DCFH oxidation was also inhibited by various cytochrome P450 inhibitors (e.g., 1-benzylimidazole, cimetidine, SKF525A). The P450 activator H_2O_2 markedly increased DCFH oxidation and was prevented by P450 inhibitors. Endogenous DCFH oxidation was not inhibited by phenelzine (a monoamine oxidase inhibitor) or by oxypurinol (a xanthine oxidase inhibitor). Hepatocyte viability after three hours incubation was not affected by these inhibitors or scavengers at the concentrations used (results not shown).

Various inhibitors for complexes of the mitochondrial electron transport chain, such as rotenone (Complex I), AA (Complex III), and cyanide (Complex IV) markedly increased hepatocyte DCFH oxidation, as did pretreatment with N_2 for 90 min. By contrast, uncouplers of oxidative phosphorylation (carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), or pentachlorophenol) inhibited DCFH oxidation (Table 2).

As shown in Table 3, hepatocyte catalyzed DCFH oxidation and hepatocyte AA-resistant respiration was increased by the addition of

Table 3. Hepatocyte "ROS" formation by oxidase substrates

Additions	DCFH oxidation (fluor. units)			AA-resistant respiration (µmol	Cytotoxicity
Incubation time:	30 min	60 min	120 min	O_2 in10min)	120 min
Control hepatocytes	66±6	76±9	88±9	<1	17±3
Peroxisomal oxidases					
+ glycolate (1mM)	85±8*	86±7	108±8*	28±3	17±2
+ glycolate (4mM)	74±8	118±12	132±15*	63±5	18±3
+ linoleate (1mM)	76±8	116±12	126±14*	23±2	14±2
+ D-alanine (4mM)	75±7	115±13	121±14*	7±1	15±2
+ hypoxanthine (4mM)	71±7	69±7	83±7	<1	14±2
+ uric acid (1mM)	51±5**	60±5	72±8	53±4	18±3
Endoplasmic reticulum oxidases					
+ L-gulonolactone (2mM)	138±12*	112±15*	95±9	7±1	22±3
Mitochondrial MAO					
+ tyramine (2mM)	79±8	91±10	128±12*	48±4	52±5*
+ phenelzine (3µM)	58±6	64±8	73±9	4±1	21±3
+ dopamine (1mM)	47±5**	58±6**	72±7	57±6	34±5*
+ phenelzine (3µM)	49±5**	56±6**	74±8	5±1	20±3
+ benzylamine (2mM)	58±6	67±5	78±9	57±5	33±3*
+ phenethylamine (2mM)	97±7*	100±9*	112±11*	42±4	24±2*
+ norepinephrine (2mM)	76±6	90±8	136±12*	16±2	22±3
Catalase inhibited hepatocytes	87±8	97±10	106±10	<1	20±2
+ glycolate (1mM)	236±36¶	355±37¶	493±55¶	44±3¶	81±5¶
+ linoleate (1mM)	185±17¶	225±24¶	292±31¶	34±3¶	29±3¶
+ D-alanine (4mM)	134±12¶	177±15¶	281±31¶	30±3¶	29±3¶
+ hypoxanthine (4mM)	135±15¶	192±18¶	173±18¶	7±1¶	28±2¶
+ uric acid (1mM)	360±32¶	350±41¶	281±32¶	70±4¶	52±4¶
+ L-gulonolactone (2mM)	180±13¶	158±17¶	160±15¶	14±1¶	34±2¶
+ tyramine (1mM)	255±19¶	180±21¶	126±14¶	60±3¶	100¶
+ dopamine (1mM)	124±14¶	144±8¶	157±11¶	74±3¶	67±5¶
+ benzylamine (2mM)	362±34¶	385±32¶	565±51¶	67±3¶	55±3¶
+ norepinephrine (2mM)	271±25¶	283±25¶	296±28¶	44±3¶	42±3¶

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Hensleit buffer, pH 7.4 at 37°C under 10% O_2, 5% CO_2, 85% N_2. DCFH oxidation to fluorescent dichlorofluorescein was used to determine "ROS" formation as described in Materials and Methods. Cytotoxicity was determined as the percentage of cells taking up trypan blue. AA-resistant respiration was measured 10 minutes after addition of substrate so as to compare the effectiveness of the various oxidase substrates at generating intracellular H_2O_2. Values are expressed as the means of three separate experiments (±SD), where the accepted level of significance was $p < 0.05$.

* Significantly greater than control hepatocytes.

** Significantly less than control hepatocytes.

¶ Significantly greater than catalase inhibited hepatocytes.

non-cytotoxic concentrations of glycolate, a substrate for glycolate oxidase located in the peroxisomes. However, inactivation of catalase, also located in the peroxisomes, markedly increased glycolate induced DCFH oxidation and resulted in cytotoxicity. Other substrates of peroxisomal oxidases such as linoleate, a substrate for fatty acyl-CoA oxidase, D-alanine, a substrate for D-amino acid oxidase, or uric acid, a substrate for uricase, also increased AA-resistant respiration in control hepatocytes but were not cytotoxic. However, AA-resistant respiration and DCFH oxidation markedly increased cytotoxicity, when these peroxisomal substrates were added to catalase inhibited hepatocytes.

Substrates of mitochondrial monoamine oxidase (MAO) such as tyramine, phenethylamine or norepinephrine also increased DCFH oxidation and cytotoxicity

ensued. Furthermore, the MAO substrate induced DCFH oxidation and cytotoxicity was prevented by the irreversible MAO inhibitor phenelzine. Tyramine, phenethylamine or norepinephrine induced DCFH oxidation, and cytotoxicity was markedly increased in catalase inhibited hepatocytes (Table 3). However, dopamine was less effective than other MAO substrates at increasing DCFH oxidation even though it was particularly effective at inducing AA-resistant respiration and was more cytotoxic. L-Gulonolactone, a substrate for gulonolactone oxidase located in the endoplasmic reticulum, also induced DCFH oxidation particularly in catalase inactivated hepatocytes but was less cytotoxic (Table 3).

The order of effectiveness for oxidase substrates (standardized to 1mM concentrations) at inducing AA-resistant respiration was dopamine > uric acid > tyramine > glycolate, linoleic acid, benzylamine > phenethylamine > norepinephrine > D-alanine > hypoxanthine. The order of cytotoxic effectiveness for oxidase substrates in catalase inhibited hepatocytes was tyramine > glycolate > dopamine > uric acid > gulonolactone > norepinephrine. D-alanine, hypoxanthine and L-gulonolactone were not cytotoxic (Table 3).

The DCFH oxidation catalyzed by control hepatocytes is most likely mediated by "ROS" as it was inhibited by hydroxyl radical scavengers or "ROS" scavengers. It was also increased by catalase inhibitors. Reduced P450 isozymes seem to be a source of "ROS" as inhibitors of P450 isozymes inhibited hepatocyte "ROS" formation.

The catalytic mechanism for cytochrome P450s involves reductive activation of molecular oxygen by several electrons supplied by P450 reductase and NADPH (17). Uncoupling of the catalytic cycle could result in the release of O_2^- and H_2O_2 as autoxidation of the oxycytochrome P450 complex may form O_2^-, whereas H_2O_2 may be formed by the protonation and decay of the P450 peroxy complex (18). Rat liver CYP2E1 was shown to be particularly active in producing H_2O_2 and catalyzing lipid peroxidation, compared with other cytochrome P450s (19). Rat liver microsomal catalyzed DCFH oxidation was also increased in CYP2E1, CYP2B, CYP3A2 but not CYP2C11 induced microsomes (20). Recently, microsomes expressing human cytochrome P450s were shown to oxidize DCFH with the order of effectiveness being CYP3A4 > CYP1A1 > CYP1A2 ≈ CYP2B6 (21). CYP3A4 is the major cytochrome P450 in human liver microsomes and could therefore be a major "ROS" source in human liver.

It has already been shown that H_2O_2 markedly increased the cytochrome P450 catalyzed metabolic activation of xenobiotics in isolated hepatocytes (22, 23). Hepatocyte DCFH oxidation was also increased by extracellular H_2O_2 and was prevented by cytochrome P450 inhibitors. It has also been shown that H_2O_2 induced cytotoxicity to LLC-PK cells was also prevented by similar cytochrome P450 inhibitors, which did not react with "ROS" (24). H_2O_2 and cytochrome P450 may form "ROS" by releasing iron from the P450 heme and/or chelate iron (24). Alternatively, DCFH could act as a P450 ferryl donor i.e., as a peroxidase substrate for P450 as horse-radish peroxidase and H_2O_2 catalyze DCFH oxidation (9).

L-Gulonolactone oxidase is also located in the endoplasmic reticulum and produces equimolar amounts of ascorbic acid and H_2O_2 which may decrease hepatocyte GSH levels (25). Addition of L-gulonolactone to hepatocytes increased AA-resistant respiration as well as endogenous DCFH oxidation. Furthermore, L-gulonolactone induced more DCFH oxidation in catalase inhibited hepatocytes which did not result in hepatocyte cytotoxicity, possibly as a result of the scavenging of "ROS" by ascorbic acid.

Mitochondrial respiration consumes most of the oxygen used by cells and therefore some of the endogenous "ROS" formation of the hepatocytes may be attributed to autoxidation of the reduced components of the respiratory electron transport chain. "ROS" formation was markedly increased by rotenone, an inhibitor of NADH dehydrogenase (Complex I). Previously, O_2^- formation by heart submitochondrial particles was also found to be increased by rotenone and Complex I was suggested to be an important mitochondrial site of O_2^- generation (7). Antimycin A, an inhibitor of the ubiquinone-cytochrome b reductase (complex III), also stimulated "ROS"

formation. Previously, O_2^- formation by heart submitochondrial particles was also found to be increased by antimycin A, which was attributed to the reaction of O_2^- with reduced cytochrome b566 (26) and/or ubi-semiquinone (27). DCFH oxidation was also increased by keeping the hepatocytes under a nitrogen atmosphere for 90 min before restoring oxygen and adding DCFH. Similar results were previously reported using luminol chemiluminescence to follow "ROS" formation (28).

Of particular interest was the strong inhibition of endogenous "ROS" formation by isolated hepatocytes on addition of non-toxic concentrations of the uncouplers CCCP and pentachlorophenol. This suggests that mitochondria are a major contributer to endogenous "ROS" formation by isolated hepatocytes. Recently, mitochondrial uncouplers were reported to strongly inhibit H_2O_2 generation by isolated heart muscle mitochondria as well as inhibiting reverse electron transfer from succinate to NAD^+ (29). The authors suggested that mitochondrial reverse electron transfer to complex I produces H_2O_2. This inhibition of "ROS" formation by uncouplers suggests that hepatocytes *in vivo* may form less endogenous "ROS" as a result of mild physiological uncoupling by fatty acids and/or thyroxine *in vivo* (6).

The peroxisomal substrates glycolate and D-alanine increased AA-resistant respiration as well as endogenous hepatocyte DCFH oxidation which was markedly increased in catalase inhibited hepatocytes, and was sufficient in the case of glycolate to result in cell death. This suggests that the H_2O_2 formed by peroxisomal glycolate oxidase and D-amino acid oxidase is likely decomposed by the catalase located and stored in the peroxisomes (30). A similar conclusion was reached when endogenous hepatocyte H_2O_2 formation induced by glycolate was determined by following catalase complex I formation in control hepatocytes and GSSG formation in catalase inactivated hepatocytes (31). However, glycolate induced cytotoxicity in catalase inactivated hepatocytes was not investigated.

Xanthine oxidase, located in hepatocyte peroxisomes and cytosol (32) did not contribute to endogenous DCFH oxidation as there was no effect of oxypurinol, a xanthine oxidase inhibitor, or hypoxanthine, a xanthine oxidase substrate. This could indicate that xanthine dehydrogenase is mainly responsible for hypoxanthine metabolism in control hepatocytes, whereas xanthine oxidase contributes to "ROS" formation during ischemic reperfusion injury (33).

The fatty acid linoleate (substrate for the peroxisomal fatty acyl CoA oxidase) increased endogenous hepatocyte DCFH oxidation and AA-resistant respiration. Fatty acid induced DCFH oxidation was further increased in catalase inhibited hepatocytes. Previously, other investigators showed that rat liver perfusion with these fatty acids caused GSSG efflux (attributed to H_2O_2 formation) particularly if the liver peroxisomes were induced before hand by administration of nafenopin (34). In the non-induced perfused rat liver, H_2O_2 was detected as a catalase compound I complex on perfusion with fatty acids (35). Induction of peroxisomes in rodents with plasticizers, herbicides, steroids, or hypolipidemic drugs also lead to hepatic oxidative damage, lipid peroxidation (conjugated dienes, lipofuscin), hepatocyte hyperplasia/hypertrophy, and the formation of liver tumors. Under these conditions, peroxisomes occupy as much as 25% of the cytoplasmic volume instead of the normal amount (less than 2%). This sustained oxidative stress in combination with cell proliferation appears to play a critical role in hepatocarcinogenesis. The hepatocyte oxidative stress was attributed to peroxisomal fatty acyl CoA oxidase (induced 20-40 fold), endoplasmic reticular CYP4A isoforms (induced 20-40 fold) and peroxisomal uricase (induced 2-3 fold). However, peroxisomal catalase was only induced 2 fold, and this imbalance with the oxidases could result in an excessive generation of H_2O_2 within the peroxisomes (30). Total hepatocyte superoxide dismutase and GSH peroxidase were also decreased (36). Peroxisomal L-α-hydroxy acid oxidase and D-amino acid oxidase, on the other hand, were not induced. Furthermore, cell transformation occurred if cells with overexpressed peroxisomal uricase or fatty acyl CoA oxidase were continually exposed to the substrates of these oxidases (30).

Substrates of MAO, located in the outer mitochondrial membrane, also increased

cyanide resistant respiration with an order of effectiveness of dopamine > benzylamine > tyramine > phenethylamine > norepinephrine. Furthermore, MAO inhibitors e.g., phenelzine, inhibited the AA-resistant respiration and prevented cytotoxicity. However, whilst endogenous DCFH oxidation was increased by tyramine, phenethylamine > norepinephrine the oxidation of DCFH was decreased by dopamine, benzylamine. Incubation of tyramine (2mM) with rat brain mitochondria has recently been shown to result in a 10-fold rise in protein-glutathione mixed disulfides and a decrease in mitochondrial respiration (37). Dopamine was the most effective substrate at inducing AA-resistant respiration and was cytotoxic, but is also a "ROS" scavenger (38), and thus could prevent "ROS" induced DCFH oxidation. This also suggests that dopamine induced cytotoxicity could involve H_2O_2, but not "ROS", whereas the latter may contribute to the cytotoxicity of other MAO substrates.

Diphenyleneiodonium chloride also prevented endogenous hepatocyte "ROS" formation. However, its mechanism of action is not clear as diphenyleneiodonium chloride is an irreversible inhibitor of flavoenzymes that forms phenylated flavin adducts of NADPH:cytochrome P450 reductase and mitochondrial NADH dehydrogenase (Complex I) (39). Recently, the inhibition of hepatocyte endogenous "ROS" formation has been attributed to inhibition of the latter and the electron flow into Complex I before the rotenone binding site (5). However, diphenyleneiodonium chloride, under the conditions described in Table 1, inhibited hepatocyte respiration by only 20% (results not shown).

In conclusion, the oxidation of DCFH by intact cells can be used to measure the effects of xenobiotics or endobiotics on endogenous "ROS" formation. Furthermore, an indication of the intracellular target responsible for the increase or decrease of "ROS" formation induced by the xeno/endobiotic can be ascertained if combined with other measurements such as changes in cellular respiration, induction of AA-resistant respiration or decrease of mitochondrial membrane potential.

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