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NADPH-induced chemiluminescence and lipid peroxidation in kidney microsomes

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NADPH-induced chemiluminescence and lipid peroxidation in kidney microsomes. Lipid peroxidation and reactive oxygen species have been shown to affect diverse biological processes potentially important in renal disease. We therefore examined NADPH-induced chemiluminescence (CL) and lipid peroxidation (LP) by renal cortical and, in some experiments, medullary microsomes. We further examined the role of reactive oxygen species in these processes by the use of enzymatic and chemical scavengers. Cortical microsomes gave a marked NADPH-induced CL accompanied by LP. The time course of LP closely paralleled the CL response. Cortical microsomal CL and LP increased with increasing concentrations of protein (0.3 to 1.8 mg) and NADPH (0.1 to 3.0 mM); NADH could not substitute for NADPH. Using similar amounts of protein and NADPH concentrations, cortical CL was significantly higher than medullary CL at all time points examined (peak cortical CL: 490 ± 25 × 10^3 cpm/mg protein, $N = 4$; peak medullary CL: 226 ± 61 × 10^3 cpm/mg protein, $N = 4$). Cortical LP was similarly higher at all time points, values corresponding to peak CL being 44.7 ± 3 nmoles/mg protein for cortex and 29.9 ± 0.8 nmoles/mg protein for medulla. Para-chloromercuribenzoate (PCMB), an inhibitor of NADPH-cytochrome P450 reductase, caused a marked inhibition of the microsomal CL and LP whereas SKF 525A, an inhibitor of cytochrome P450, had a relatively minor effect. Marked inhibition of NADPH induced CL and LP was observed with chelators EDTA and 1,10-phenanthroline. The addition of NADPH to microsomes prepared with sucrose-EDTA and washed in a Chelex-treated buffer gave a negligible CL and LP response; the responses were restored by the addition of iron. Scavengers of superoxide anion (superoxide dismutase), hydrogen peroxide (catalase), and the hydroxyl radical (sodium benzoate, tryptophan) had a relatively minor effect on CL and LP. Singlet oxygen quenchers sodium azide and 1,4-diazabicyclo (2.2.2) octane (DABCO) caused an incomplete inhibition of CL and LP responses and then only after the first 30 min. Taken together, these data suggest that other free radical mechanisms are likely to be important in the CL and LP responses. These results which demonstrate the production of excited states and LP by renal microsomes suggest that their role in renal disease deserves further study.

Chemiluminescence induite par le NADPH et peroxydation des lipides dans les microsomes de rein. Il a été montré que la peroxydation des lipides et le type d'oxygène réactif modifient diversement les processus biologiques potentiellement importants dans les maladies rénales. C'est pourquoi nous avons examiné la chemiluminescence (CL) induite par le NADPH, et la peroxydation (LP) des lipides par des microsomes corticaux et dans quelques expériences, médullaires rénaux. Nous avons ensuite examiné le rôle du type d'oxygène réactif dans ces processus en utilisant des agents métabolisants enzymatiques et chimiques. Les microsomes corticaux donnaient une CL marquée induite par le NADPH accompagnée par une LP. Dans le temps, la LP était étroitement parallèle à la réponse CL. La CL et la LP microsomiales corticales s'élevaient avec l'accroissement des concentrations de protéines (0, 1 à 1,8 mg) et de NADPH (0,1 à 3,0 mM); le NADH ne pouvait se substituer au NADPH. En utilisant des quantités identiques de protéines et de NADPH, la CL corticale était significativement plus élevée que la CL médullaire à tous les temps examinés (pic de CL corticale: 490 ± 25 × 10^3 cpm/mg protéine, $N = 4$, pic de CL médullaire: 226 ± 61 × 10^3 cpm/mg protéine, $N = 4$). De même la LP corticale était plus élevée à tous les temps, les valeurs correspondant au pic de CL étant de 44,7 ± 3 nmoles/mg protéine pour le cortex et de 29,9 ± 0,8 nmoles/mg protéine pour la médullaire. Le parachloromercuribenzoate (PCMB), un inhibiteur de la réductase NADPH-cytochrome P450 a entraîné une inhibition marquée de la CL et de la LP microsomiales tandis que le SKF 525 A, un inhibiteur du cytochrome P450, avait un effet relativement minime. Une inhibition marquée de la CL induite par le NADPH et de la LP a été observée avec des chélateurs tels l'EDTA et le 1,10-phénanthroline. L'addition de NADPH à des microsomes préparés dans du sucrose-EDTA et lavés dans un tampon traité au Chelex entraînait une réponse CL et LP négligeable; les réponses étaient restaurées par l'addition de fer. Les agents métabolisants l'anion superoxide (dismutase superoxide), le peroxide d'hydrogène (catalase), et le radical hydroxylé (benzoate de sodium, tryptophane) avaient un effet relativement minime sur CL et LP. Les capteurs d'oxygène singulet comme l'azide de sodium et le 1,4-diazabicyclo (2.2.2) octane (DABCO) entraînaient une inhibition incomplète des réponses CL et LP et cela seulement après les 30 premières min. Prises dans leur ensemble, ces données suggèrent que d'autres mécanismes par radicaux libres sont probablement importants dans les réponses CL et LP. Ces résultats, qui démontrent la production d'états excités et de LP par des microsomes rénaux, suggèrent que leur rôle dans les maladies rénales nécessite d'autres études.

Biological membranes have a high content of polyunsaturated fatty acids and in addition are constantly being bathed in oxygen rich fluids. As a result, they are particularly susceptible to peroxidative attack by oxygen-derived free radicals, resulting in lipid peroxidation (LP) [1–3]. The oxygen-derived free radicals and lipid peroxidation can alter membrane structure [1, 2, 4] and permeability [1, 2] and affect activities of several membrane-bound enzymes [1, 2, 5–8]. In addition LP and reactive oxygen species can affect a variety of other biological processes potentially important in renal disease [9–18].

Lipid peroxidation has been shown to occur both in vivo [19, 20] and in vitro in a variety of tissues and subcellular fractions

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including nuclei, lysosomes, mitochondria, and microsomes [1, 2, 9, 21–24]. The endoplasmic reticulum (isolated as microsomes) is among the best characterized and most active subcellular fractions with respect to LP. Microsomes catalyze an iron and NADPH-dependent peroxidation of endogenous lipid [25]. This NADPH-induced LP, sometimes referred to as enzymatic LP, is associated closely with the microsomal electron transport chain which catalyzes oxidative drug metabolism [26]. Both the LP and drug metabolizing systems utilize the enzyme NADPH-cytochrome P450 reductase [27, 28].

Several studies have shown the production of reactive oxygen species during NADPH-induced LP [1, 2, 29–33]. These reactive oxygen species, including superoxide anion, hydroxyl radicals, and singlet oxygen, have been implicated in both the initiation and propagation of LP [1, 2, 34–39].

The addition of NADPH to microsomes also produces a chemiluminescence (CL) indicative of the presence of excited states, which, upon relaxation to ground state, emit light. This low level of light emission can be measured in a liquid scintillation counter. Because of the low energy of the light emitting species, the liquid scintillation counter must be operated in the out-of-coincidence mode which allows events from both the photomultiplier tubes to be seen individually and summed [40]. The precise mechanisms responsible for this CL are not known. Originally Allen, Stjernholm, and Steele [41] attributed the CL from phagocytic cells to singlet oxygen. However, recent studies indicate, that at least in the CL response of phagocytic cells, several reactive oxygen species including superoxide anion, hydroxyl radical, hydrogen peroxide, and possibly singlet oxygen are involved [42–44]. In NADPH-induced microsomal CL, such reactive oxygen species may also be partly responsible for the observed CL. In addition, however, reactive lipid intermediates appear to be important in NADPH-induced microsomal CL. A close association between microsomal CL and LP appears to exist [2, 45] and, in fact, CL has been used as an index of LP [46, 47].

NADPH-induced CL, its relationship to LP, and the role of reactive oxygen species in these processes in renal microsomes has not been examined previously. We therefore examined NADPH-induced chemiluminescence and lipid peroxidation in renal cortical microsomes and in some experiments medullary microsomes obtained from rat kidney. We further examined the role of reactive oxygen species in these processes by utilizing several scavengers of reactive oxygen species.

Methods

Kidneys were rapidly removed from male Sprague-Dawley rats and placed in ice-cold 0.25 M sucrose. All subsequent manipulations were made at 0 to 4°C. Each cortex and medulla was separated carefully, weighed, minced and homogenized in 0.25 M sucrose (1:5 wt/v) using a Potter-Elvehjem smooth glass homogenizer with a motor driven pestle (Teflon). The microsomal fraction was obtained by differential centrifugation, essentially as described in our previous study [48]. The microsomal pellet was washed once with 0.1 M phosphate buffer, pH 7.4, then resuspended in the same buffer, divided into several small aliquots, and stored at −80°C.

Chemiluminescence measurements were carried out at ambient temperature, in a dark room illuminated with red light. Incubations were carried out in dark adapted glass scintillation vials. CL was measured with a liquid scintillation system (Beckman Instruments Inc., Fullerton, California), operated in the out-of-coincidence summation mode as described in our previous studies [48, 49]. A standard reaction mixture consisted of 0.2 ml microsomal preparation (1 to 2 mg protein) and sufficient 0.1 M phosphate buffer pH 7.4 so that the final volume after all additions was 3.0 ml. Under these conditions the CL of vials containing only buffer and microsomes ranged between 5,000 to 10,000 cpm. In our initial experiments, we observed, as have other investigators [25, 45, 50] that the addition of exogenous iron was not necessary for NADPH-induced CL and LP. It has been suggested that even small amounts of iron present in microsomes and/or phosphate buffer are sufficient for LP [25, 45, 50]. Reactions were initiated by the addition of NADPH (Sigma Chemical Company, St. Louis, Missouri) with a final concentration of 3 mM for most experiments, and CL was measured at different time intervals as shown in the **Results**. In every experiment vials without added NADPH were also included.

Malondialdehyde production, which has been used extensively to quantify LP, was measured using the thiobarbituric acid assay of Ottolenghi [51]. In some experiments (Figs. 1, 2, and 3) 0.5-ml aliquots were obtained at the end of the incubation from the same vials as those in which CL measurements were made. In all other experiments, aliquots were obtained at several time points from incubations which were run in parallel with the incubations for CL measurements; the aliquots were obtained at the same time points as the CL measurements. Each 0.5-ml aliquot was added to 1 ml of 17.5% TCA to terminate the reaction and stored at −20°C for subsequent measurement of malondialdehyde. Malondialdehyde was determined as follows: One milliliter of 0.6% (w/v) 2-thiobarbituric acid was added to thawed samples, and after thorough mixing, the tubes were placed in a boiling water bath for 15 min. After cooling (in a water bath at ambient temperature), 1 ml of 70% trichloroacetic acid was added, and samples were allowed to stand for 20 min at room temperature. The samples were then centrifuged and the optical density of the clear supernatants determined at 535 nm against a reagent blank. The amount of malondialdehyde, expressed in nanomoles, was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} at 535 nm [45, 52]. Values for CL and LP obtained in the absence of NADPH were subtracted to obtain NADPH-induced CL and LP. CL measurements are expressed as cpm/mg protein and malondialdehyde production as nmoles/mg protein. Protein determinations were carried out by the Bio-Rad method (Bio-Rad Lab, Richmond, California) as described by the supplier.

The role of reactive oxygen species in CL and LP was examined by using enzymatic and chemical scavengers. Superoxide dismutase (type 1), catalase (thymol free), benzoate, trytophan, histidine, and sodium azide (all obtained from Sigma Chemical Company, St. Louis, Missouri) and 1,4-diazabicyclo [2.2.2]octane (DABCO) (Aldrich Chemical Co., Milwaukee, Wisconsin) in concentrations similar to those used by others [42, 43, 53, 54] were added to the vials prior to addition of NADPH (Table 2, Fig. 5). The effect of the sulfhydryl agent, p-chloromercuribenzoate (PCMB) (Sigma Chemical Company), an inhibitor of NADPH-cytochrome P450 reductase [55, 56] and SKF525A, (a generous gift from Smith Kline and French Laboratories, Philadelphia, Pennsylvania) an inhibitor of cyto-

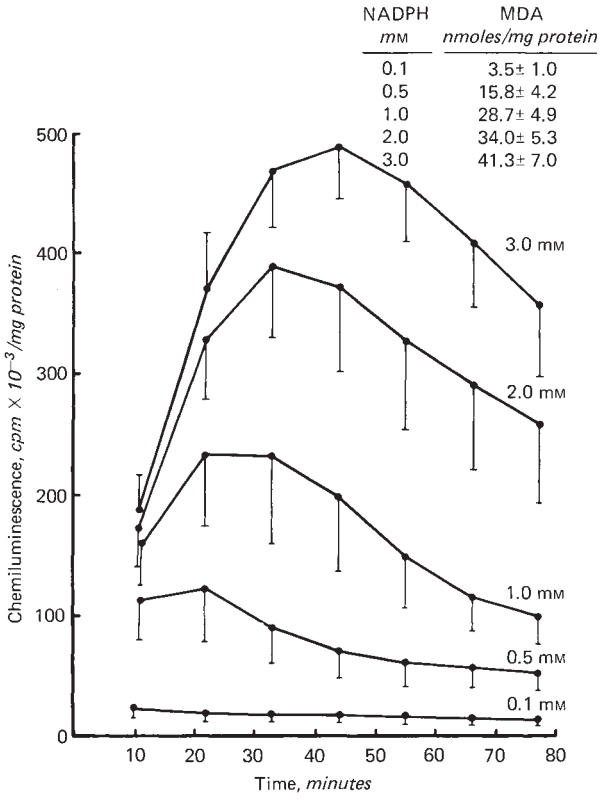

Fig. 1. *Effect of NADPH concentration on cortical microsomal chemiluminescence and lipid peroxidation.* A standard reaction mixture consisted of 0.2 ml microsomal preparation (1 to 2 mg protein) and sufficient 0.1 M phosphate buffer, pH 7.4, so that the final volume after all additions was 3.0 ml. The reaction was initiated by the addition of NADPH (final concentrations shown) and chemiluminescence measured at different time intervals. Lipid peroxidation (*insert*) was quantified by measuring malondialdehyde (MDA) production in aliquots of reaction mixtures obtained at the end of the incubations. Results shown are the mean ± SEM from three separate experiments.

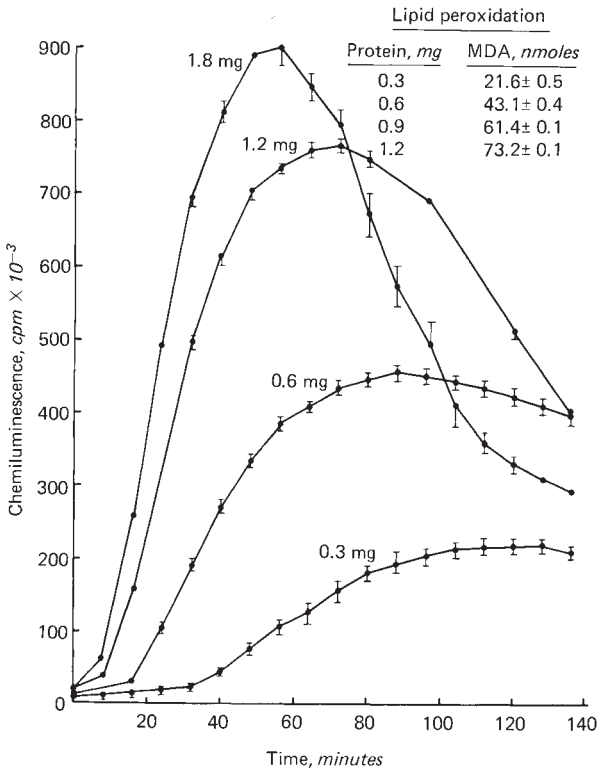

Fig. 2. *Effect of protein concentration on NADPH-induced cortical microsomal chemiluminescence and lipid peroxidation.* Results (mean ± SEM) of one experiment performed in triplicate are shown. Similar results were obtained in two other experiments performed in triplicate. See the legend to Figure 1 and **Methods** for details.

chrome P450 [57], and chelators EDTA and 1,10-phenanthroline (Eastman Kodak, Rochester, New York) on NADPH-induced CL and LP were also examined (Table 1). The effects of these agents and scavengers on CL and LP are expressed as a percentage change compared to control vials containing the same microsomes incubated under identical conditions. The percentage change was calculated as follows:

$$\frac{\text{Value in experimental vials} - \text{Value in control vials}}{\text{Value in control vials}} \times 100.$$

In these experiments, the effects of these agents on CL were measured at several time points; the effects on LP were measured at the same time in parallel incubations. For those experiments in which the observed effects were similar at various time points, only the effect at the time of peak CL response is shown (Tables 1 and 2).

To demonstrate the dependence of NADPH-induced CL and LP on iron, renal cortical microsomes were prepared from three separate groups of rats using 0.25 M sucrose in 1 mM EDTA (pH 7.4). These microsomes were washed in Chelex-100-treated 0.1 M phosphate buffer, pH 7.4, to remove EDTA [25], and resuspended in the same buffer. For these experiments only glassware (including homogenizer tubes and scintillation vials), previously rinsed with 50% nitric acid, was utilized. The incubation conditions were the same as other experiments except that Chelex-100-treated phosphate buffer was used. The effect of NADPH (3 mM) with and without added iron on microsomal CL and LP was examined. The effect of iron by itself (without NADPH) was also examined. As described by others [22, 30], iron was added as ADP-Fe^{3+} (ADP 1 mM, $FeCl_3$ 0.01 mM), with ADP apparently as an aid to maintain iron in the solution [30].

Results

In the absence of NADPH, there was negligible CL (5,000 to 10,000 cpm) and malondialdehyde production (0.4 to 2.1 nmoles/mg protein) by microsomes. In the data presented these values have been subtracted to calculate NADPH-induced CL and LP. The time course of CL resulting from the addition of different concentrations of NADPH to cortical microsomes is shown in Figure 1. Negligible CL was observed with 0.1 mM NADPH. Increasing concentrations of NADPH gave a higher CL response at every time point; the highest response was observed with 3 mM NADPH. Malondialdehyde production, measured in the same samples at the end of the incubations, also increased with higher concentrations of NADPH (Fig. 1). NADH (1 mM or 3 mM) could not substitute for NADPH in inducing either the CL or LP response.

Table 1. Effect of p-chloromercuribenzoate, SKF525-A, and chelators on NADPH-induced chemiluminescence and lipid peroxidation in renal cortical microsomes[a]

	% Change at the time of peak CL response[b]	
	Chemiluminescence	Lipid peroxidation
PCMB, *1 mM*	−99 ± 0.03 (3)[c]	−97 ± 0.3 (3)
SKF525-A, *1 mM*	−5 ± 2.0 (3)	−12 ± 0.5 (3)
EDTA, *1 mM*	−99 ± 0.2 (3)	−77 ± 1.6 (3)
1, 10-Phenanthroline, *1 mM*	−94 ± 3.4 (3)	−57 ± 3.4 (3)

[a] NADPH-induced chemiluminescence was measured at different time points as described in **Methods** and shown in Figure 5. Lipid peroxidation, quantified by malondialdehyde production, was measured in aliquots obtained from parallel incubations; the aliquots were obtained at the exact time points as the chemiluminescence measurements. Results are shown at the time of peak chemiluminescence; similar results were obtained at all other time points.

[b] The percentage change was calculated as follows:

$$\frac{\text{Value in presence of inhibitor} - \text{Control value}}{\text{Control value}} \times 100.$$

[c] All values are mean ± SEM. The numbers in parentheses indicate the number of experiments.

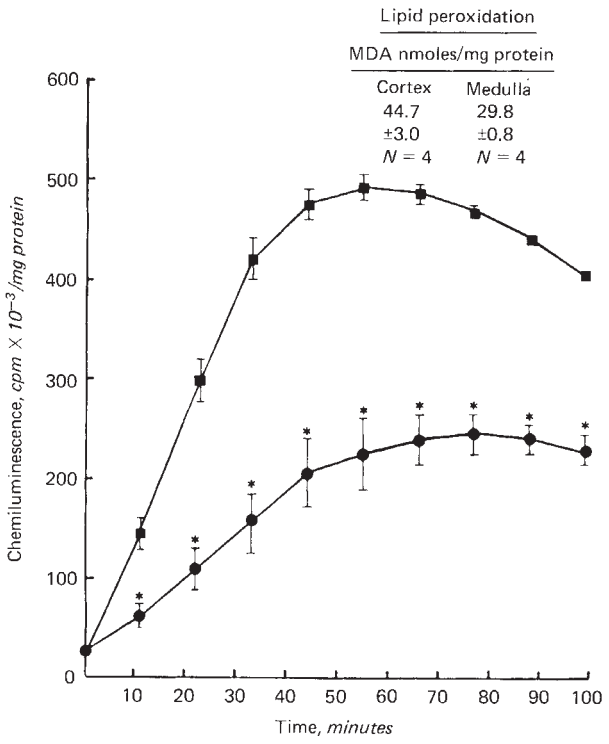

Fig. 3. *NADPH-induced chemiluminescence and lipid peroxidation in cortical and medullary microsomes.* Cortical and medullary microsomes were obtained at the same time from the same rats, and incubations were carried out simultaneously using 3 mM-NADPH and similar amounts of microsomal protein. The *insert* shows the maldialdehyde (MDA) production in the same samples at the end of the incubations. Symbols are: ■——■, cortex; ●——●, medulla. All results shown are the mean ± SEM of four separate experiments in triplicate. *Asterisks* denote $P < 0.05$ or a greater level of significance. See the legend to Figure 1 and **Methods** for details.

The effect of protein concentration on the NADPH-induced CL and LP is shown in Figure 2. The higher the microsomal protein concentration, the higher and earlier was the maximum CL response. The amount of malondialdehyde formed also increased with higher protein concentrations (Fig. 2). The results depicted in Figure 2 are from one experiment performed in triplicate; however, similar results were obtained in two other experiments.

The sulfhydryl reagent, p-chloromercuribenzoate (1 mM), an inhibitor of NADPH cytochrome P450 reductase [55, 56] caused a marked inhibition of both CL and LP in renal cortical microsomes. At the time of peak CL response, CL was inhibited −99 ± 0.03% ($N = 3$), and LP −97 ± 0.3% ($N = 3$) (Table 1). Inhibitory effects were similarly observed at all other time points (data not shown). In contrast, SKF 525A (1 mM), an inhibitor of cytochrome P450 [57], had only a minor effect on both NADPH-induced CL and LP at all time points examined. At the time of peak CL response, CL was inhibited −5 ± 2%, ($N = 3$) and LP −12 ± 0.5%, ($N = 3$) (Table 1). Both chelators EDTA (1 mM) and 1,10 phenanthroline (1 mM) caused marked inhibition of NADPH-induced CL and LP at all time points (Table 1). Addition of NADPH to cortical microsomes prepared with sucrose-EDTA gave only a negligible response at all time points examined (peak CL: 19 ± 1 × 10^3 cpm/mg protein; LP: 2.8 ± 0.4 nmoles/mg protein, $N = 3$). When ADP-Fe^{3+} (ADP 1 mM; $FeCl_3$ 0.01 mM) was included in the incubations in concentrations similar to those used by others [22, 30] a marked NADPH-induced response was observed (peak CL: 398 ± 9 × 10^3 cpm/mg protein; LP: 19.8 ± 1.6 nmoles/mg protein, $N = 3$). Addition of ADP-Fe^{3+} alone gave a negligible response similar to that observed in microsomes without NADPH (CL: 0.1 ± 0.1 × 10^3 cpm/mg protein; LP: 3.0 ± 0.5 nmoles/mg protein, $N = 3$).

We compared the NADPH-induced CL response and LP in cortical and medullary microsomes. These microsomes were prepared simultaneously from the same animals, and the incubations were carried out in the same experiments. Based on the data presented above, we used 3 mM NADPH and similar amounts of protein for cortical and medullary microsomes. The results of these experiments are shown in Figure 3. At every time point, the CL response was higher in cortical microsomes compared to medullary microsomes. Correspondingly, malondialdehyde production, measured in the same vials at the end of incubation, was significantly higher in cortical microsomes (44.7 ± 3.0 nmoles/mg protein, $N = 4$) compared to medullary microsomes (29.8 ± 0.8 nmoles/mg protein, $N = 4$).

To study the relationship between CL and LP, the two responses were measured at various time points in the same microsomal preparations in simultaneous incubations (Fig. 4). A close relationship between CL and LP was observed, both responses reaching a peak at about 40 to 50 min. Additionally in experiments to determine the effect of protein concentration on CL and LP, the peak CL response was correlated with LP. In three separate experiments, each with four different protein concentrations in triplicate, the peak CL showed a significant correlation with LP, with correlation coefficients of 0.97, 0.92, and 0.94.

We next evaluated the role of reactive oxygen species in the CL and LP responses by using scavengers of reactive oxygen species. In these experiments the effects of the scavengers on

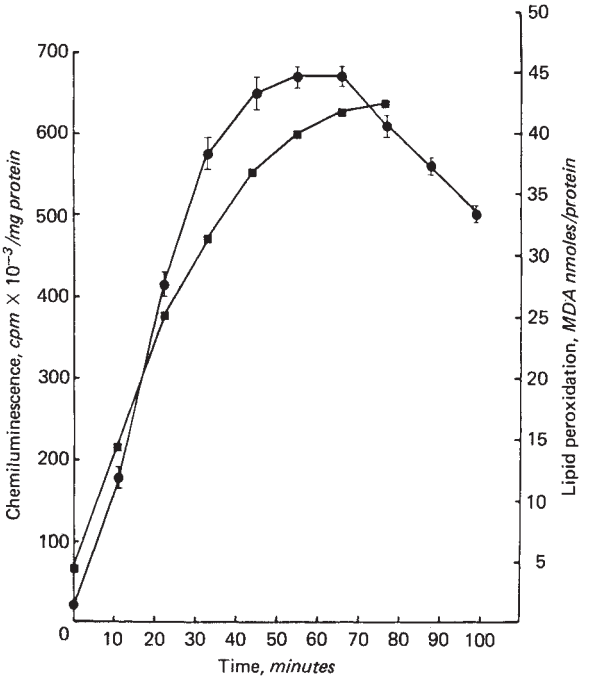

Fig. 4. *Time course of NADPH-induced chemiluminescence and lipid peroxidation in cortical microsomes.* The reaction was initiated by the addition of 3 mM NADPH and chemiluminescence was measured at different time intervals as shown. For lipid peroxidation (■——■), parallel incubations were run and aliquots for measurement of malondialdehyde (MDA) were obtained at exactly the same time points as chemiluminescence (●——●) measurements. Values shown are mean ± SEM of three separate experiments performed in triplicate. See the legend to Figure 1 and **Methods** for details.

CL were measured at several time points, with LP measurements obtained at the same time from parallel incubations. Since similar effects were observed at all time points with the scavengers (except DABCO), only the effect at the time of peak CL is shown (Table 2). Superoxide dismutase, an enzyme that scavengers superoxide anion, in two different doses caused a consistent but small increase in both CL and LP (Table 2). Inactivated superoxide dismutase (100°C × 15 min) caused a small inhibitory effect on CL (−13 ± 0.3%, $N = 3$) and LP (−6 ± 0.2%, $N = 3$). We also examined the effect of catalase, an enzyme which destroys hydrogen peroxide, on CL and LP. Catalase (with thymol, an antioxidant, as a preservative) caused a marked inhibition of CL (−91 ± 5%, $N = 3$). However, thymol by itself also had a marked inhibitory effect on CL (−93 ± 5%, $N = 3$). When thymol-free catalase was used, only a small inhibitory effect on CL and LP was noted (Table 2). Bovine serum albumin in concentrations similar to those used for catalase caused a small inhibitory effect on CL (−11 ± 0.3%, $N = 3$) and LP (−3 ± 0.3%, $N = 3$). Two hydroxyl radical scavengers, sodium benzoate and tryptophan, in doses similar to those used by other investigators [42, 53], also produced a relatively minor inhibition of the CL and LP (Table 1). The effects of singlet oxygen quenchers were as follows: After a small lag, histidine had a relatively minor effect on CL and LP (data not shown). Azide caused inhibition of NADPH-induced CL and LP response, and the maximum inhibitory effects were observed after the first 30 min. At the time of peak CL, CL was

Table 2. Effect of scavengers of reactive oxygen species on the NADPH-induced chemiluminescence and lipid peroxidation by renal cortical microsomes[a]

	% Change at the time of peak CL response[b]	
	Chemiluminescence	Lipid peroxidation
Superoxide dismutase[c]		
200 μg/ml	+8 ± 2 (4)[d]	+12 ± 3 (4)
400 μg/ml	+9 ± 1 (4)	+10 ± 2 (4)
Catalase, *250 μg/ml*[c]	−14 ± 2 (4)	−11 ± 2 (4)
Sodium benzoate, *20 mM*	−6 ± 1 (4)	−9 ± 3 (4)
Tryptophan, *1 mM*	−3 ± 1 (4)	−11 ± 2 (4)
Sodium azide, *1 mM*	−60 ± 3 (4)	−31 ± 4 (4)

[a] NADPH-induced chemiluminescence was measured at different time points as described in **Methods** and shown in Figure 5. Lipid peroxidation as quantified by malondialdehyde production was measured in aliquots obtained from parallel incubations; the aliquots were obtained at the exact time points as the chemiluminescence measurements. Results are shown at the time of peak chemiluminescence; similar results were obtained at all other time points.

[b] The percentage change was calculated as follows:

$$\frac{\text{Value in presence of scavenger} - \text{Control value}}{\text{Control value}} \times 100$$

[c] Heat-inactivated superoxide dismutase and bovine serum albumin had a minor inhibitory effect on both CL and LP (see **Results**).

[d] All values are mean ± SEM. The numbers in parentheses indicate the number of experiments.

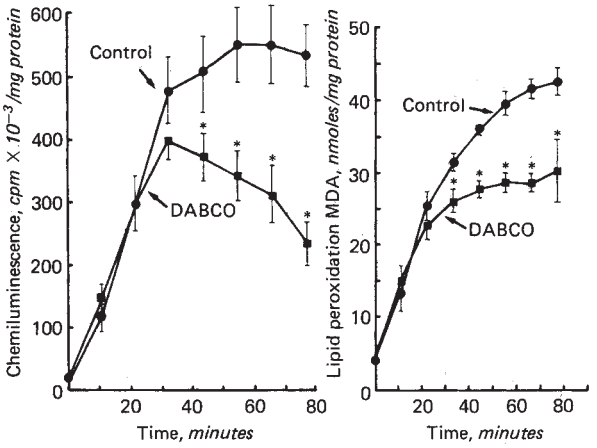

Fig. 5. *Effect of DABCO (20 mM) on NADPH-induced chemiluminescence* (left panel) *and lipid peroxidation* (right panel) *in cortical microsomes.* Values shown are mean ± SEM from four separate experiments performed in triplicate. *Asterisks* denote $P < 0.05$ or a greater level of significance. See the legend to Figure 1 and **Methods** for details.

inhibited −60 ± 3%, $N = 4$, and LP −31 ± 4%, $N = 4$ (Table 2). DABCO had no significant effect on CL and LP for the first 30 min of incubation; however, at subsequent time points, a significant inhibitory effect was observed (Fig. 5).

Discussion

NADPH-induced chemiluminescence in renal microsomes, its relationship to lipid peroxidation, and the role of reactive

oxygen species in these processes has not been examined previously. Our results show that addition of NADPH to renal cortical microsomes gave a CL response which reached a peak at about 40 to 50 min with a gradual decline thereafter. Addition of NADPH to renal cortical microsomes also resulted in LP as measured by malondialdehyde production; the time course of LP closely paralleled the CL response (Fig. 4). The NADPH-induced CL and LP responses were dose-dependent; the maximum responses were observed with 3 mM NADPH, the highest concentration tested. NADH could not substitute for NADPH.

We did not examine in detail the mechanisms responsible for the NADPH-induced CL and LP. However, in our preliminary studies we found that p-chloromercuribenzoate, a sulfhydryl reagent which inhibits NADPH-cytochrome P450 reductase [55, 56], caused a marked inhibition of both the CL and LP in renal cortical microsomes. In addition, the lack of response to NADPH by microsomes prepared with sucrose-EDTA, restoration of the response by addition of iron in the incubation medium, and the marked inhibitory effects of chelators EDTA and 1,10-phenanthroline all lend strong support for the iron requirement for NADPH-induced CL and LP. These results are in keeping with previous studies and suggest that NADPH-induced CL and LP responses are an iron-dependent [25, 50] and an enzymatic process utilizing NADPH-cytochrome P450 reductase [27, 28]. SKF 525A, an inhibitor of cytochrome P450, caused only a minor inhibition of CL and LP in cortical microsomes. This, along with our observation that medullary microsomes, which have been reported to lack cytochrome P450 [58, 59], gave a NADPH-induced response which suggests that either cytochrome P450 is not essential or that small amounts of cytochrome P450 are sufficient for NADPH-induced CL and LP [60]. Alternatively, other hemoproteins may substitute for cytochrome P450.

Using similar amounts of protein and NADPH concentrations, we found that cortical microsomes had a higher CL response and greater malondialdehyde production compared to medullary microsomes prepared simultaneously from the same rats and incubated in the same experiments (Fig. 3). The higher NADPH-cytochrome P450 reductase activity in cortex compared to medulla [59] and a recent preliminary report [58] that the highest activity of the enzyme was present in the proximal tubule may explain the higher response in the cortex. The higher cortical response may also be due to differences in the content of antioxidants as well as the amount and the nature of polyunsaturated fatty acids present in cortical and medullary microsomes.

The production of several reactive oxygen species, including superoxide anion, hydroxyl radical, and singlet oxygen during LP, has been well demonstrated [1, 2, 29–33]. These reactive oxygen species have been implicated both in initiation and propagation of LP [1, 2, 34–39]. The role of these reactive oxygen species in LP has been best demonstrated in studies utilizing either a superoxide anion generating system (for example, xanthine/xanthine oxidase) or purified NADPH cytochrome P450 reductase with exogenous lipids. In these systems several scavengers of reactive oxygen species, including superoxide dismutase, catalase, scavengers of hydroxyl radicals, and singlet oxygen, inhibited LP [34–37]. In intact microsomes with NADPH-induced LP of endogenous lipids, results with scavengers of reactive oxygen species have been conflicting [7, 22, 45, 61]. This may be due, at least partly, to a lack of accessibilities of the scavengers to the site of production of reactive oxygen species [7, 22, 61]. In our study with intact microsomes we found only minor effects of superoxide dismutase, catalase, and hydroxyl radical scavengers on CL and LP. These results are similar to those reported by others using intact microsomes [22, 45, 61]. However, as stated above, this does not entirely exclude a role for superoxide, hydrogen peroxide, and hydroxyl radical in the CL and LP in renal cortical microsomes. In addition, although the contamination with catalase in washed microsomes is likely to be minor [62], we cannot exclude the possibility that the lack of effect of exogeneously added catalase was due to a sufficient amount of catalase present in the microsomes. Previous studies have demonstrated both the production of singlet oxygen during NADPH-induced LP [30, 32] and the ability of singlet oxygen to induce LP [34–38]. In our study, histidine, a singlet oxygen quencher, had a relatively minor effect on CL and LP, similar to the effect on LP in liver microsomes [62]. DABCO and azide, also reported to be singlet quenchers, had no significant effect for the first 30 min; an incomplete inhibition of the CL and LP response was noted at subsequent time points. These results suggest that other free radical mechanisms [3] are likely to play an important role in the NADPH-induced CL and LP response by renal microsomes.

There appears to be a close relationship between CL and LP in renal cortical microsomes. As described above, the time course of CL closely paralleled the time course of LP (Fig. 4). In addition, the peak CL correlated well with LP, in experiments to determine the effect of protein concentration on CL and LP (see **Results**). Further, PCMB and chelators which markedly inhibited LP, also markedly inhibited CL whereas SKF525A had a relatively minor effect on both CL and LP. Finally, in experiments with scavengers of reactive oxygen species, the effect on CL and LP was qualitatively always in the same direction. Our results are similar to those in liver microsomes and mitochondria where a close relationship between CL and LP was observed [2, 45]. Because of this close relationship, CL has been used as a sensitive index to quantitate LP in whole organs in situ [46, 47]. However, it must be pointed out that, under special incubation conditions, CL from microsomes may be observed without accompanying LP as measured by malondialdehyde production [48].

Although LP has been documented extensively in vitro, its occurrence and hence its importance in vivo were questioned. Recent studies demonstrating the occurrence of LP in vivo [19, 20, 46, 47], coupled with diverse biological effects of LP and reactive oxygen species, suggest their possible importance in pathophysiology. Thus LP and/or reactive oxygen species have been shown to affect membrane structure [1, 2, 4] and membrane permeability [1, 2], behave as calcium ionophores [14], activate adenylate cyclase [6], increase cellular proliferation [2], and alter vascular permeability [10, 12, 13], lysosomal stability [9], and prostaglandin synthesis [17, 63]. Several observations more directly related to renal disease further support a potential role for LP and reactive oxygen species in renal pathophysiology. LP has been shown to inhibit Na-K-ATPase [5], an enzyme postulated to be important in at least one model of acute renal failure [64]. In analgesic nephropathy, the presence of tubular lipofuscin pigment [16], indicative of in vivo LP, and depletion of renal glutathione [15, 65], a reducing

agent which protects against free radical damage, is consistent with a mechanism involving oxidative damage and LP [15, 16, 65]. Both gentamicin and LP cause similar changes in mitochondrial states 3 and 4 respiration suggesting the potential involvement of lipid peroxides in gentamicin nephrotoxicity [66, 67]. These observations along with our results suggest that the role of reactive oxygen species and LP in renal pathophysiology should be examined critically.

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