Review Article

CHEMILUMINESCENCE IN THE MEASUREMENT OF FREE RADICALS: THEORY AND APPLICATION ON A TISSUE INJURY MODEL

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ABSTRACT

Reactive oxygen species have been implicated in the pathogenesis of several human diseases. However, it is difficult to quantitate reactive oxygen metabolites because of their reactive nature and short half lives. Instead, analyses of secondary or end products produced by the attack of free radicals on lipids, proteins or other cellular components are preferred. These indirect methods usually give misleading results due to their poor specificity and sensitivity. Chemiluminescence (CL) is the production of light generated from chemical sources. It may be utilized as a direct noninvasive method for measuring reactive oxygen species. Different probes with different selectivities can be used in CL measurements. The use of CL measurements in quantitating free radical reactions, advantages over other methods and its applications on a tissue injury model will be presented.

Key Words: Chemiluminescence, free radicals, pancreatitis

INTRODUCTION

One can define a free radical as any species that has one or more unpaired electrons (i.e. O_2.- NO, singlet oxygen). This definition embraces the hydrogen atom, most transition metal ions and the oxygen molecule. Transition metal ions play an important role in generation of several reactive species like H_2O_2 and .OH by their ability to participate in one-electron transfer reactions (1,2).

Reactive oxygen species (ROS) have been implicated in the pathogenesis of several human diseases (1,3,4). Increased appreciation of occurrence of ROS, their injury potential, and pathogenic role in several disease states demands quantitative methods which are diagnostic of the process and meet basic analytical criteria regarding accuracy, reliability, sensitivity, and specificity. However, it is difficult to quantitate reactive oxygen metabolites because of their reactive nature and short half lives. Instead, analyses of secondary or end products produced by free radical attack on lipids, proteins or other cellular components are preferred. These indirect methods usually give misleading results due to their poor specificity and sensitivity (1,3). Therefore, introduction of more reliable methods are necessary for accurate quantitation of ROS.

CHEMILUMINESCENCE TECHNIQUE

Chemiluminescence (CL) is the production of light generated from chemical sources. It may be utilized as a direct noninvasive method for measuring ROS. CL is a universal property of organic substances able to undergo an oxidative reaction sufficiently exothermic to produce an emitting state. Generally the light produced is visible and has a wavelength between 400-600 nm. However, this light could also be in UV or infrared region where it woud have a shorter or longer wavelength than the visible light, respectively (5).

In the 1960s several Soviet investigators reported studies of "dark chemiluminescence" from living tissues (6). Boveris et al. (7) have further summarized the current knowledge of ultraweak CL as caused by lipid peroxides, and have concluded that the sources of emission are the relaxation of singlet oxygen ($^1O_2$) to triplet ground state ($^3O_2$) and the relaxation of excited state carbonyl groups to ground state. In 1971, Howes and Steele (8) reported CL generated

by rat liver microsomes incubated with NADPH and oxygen. They also proposed that $^1O_2$ was involved in the CL. In 1972, workers from the same laboratory found that CL was generated by PMNL stimulated by bacteria (9). Due to limitations, i.e., potential variability and low intensity of native CL, various investigators introduced the use of enhancer compounds, luminol, followed more recently by lucigenin (10-13). These compounds were selected primarily because of their high quantum efficiency (high photon yield) after oxidation. When added to an *in vitro* biological system, luminol and lucigenin function as bystander-substrates for oxygenation and form high levels of excited-state products and CL. The first published work in chemically assisted CL was by Allen and Loose (10) where luminol was used as the molecule to measure PMNL function. This resulted in a system that was more than 1000-fold greater in sensitivity than native CL.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridiniumnitrate) react with oxidants, such as ROS, to form 3-aminophthalate and N-methylacridone, respectively (14). The excited electrons in these compounds revert to their ground state with the emission of energy as light (CL), which can be detected by the photomultiplier tubes of a scintillation counter. For low-level light detection there is, at present, no alternative to photomultiplier tubes. An important specification of photomultiplier tube is quantum efficiency. Photons hitting the photocathodes release single photoelectrons. Quantum efficiency is the ratio of photoelectrons released to the number of photons incident on the cathode area. The cathode type mostly used is bialkali, which is common in liquid scintillation counters (LSC). Bialkali photocathodes have excellent quantum efficiency around 400 to 450 nm and low noise, even at room temperature. This is very adequate for luminol and lucigenin emission. LSCs are designed to count events of nuclear decay by bundles of several hundred photons per disintegration. To minimize background caused by thermal noise in the detector and by single photons, the sample is measured by two multipliers and the background isfiltered off by so-called coincidence circuit, counting only events which send photons at the same time to both detectors. However, LSCs set in out-of-coincidence mode are used to measure cellular CL. This maneuver allows the instrument to measure single photon events (5).

The two CL probes, luminol and lucigenin, differ in selectivity. Luminol detects H_2O_2, OH, hypochlorite, peroxynitrite, and lipid peroxy radicals, whereas lucigenin is particularly sensitive to superoxide radical (15,16). The specificity of these techniques has been previously established (16). ROS release can be also assessed using a variety of methods, including spin trapping techniques, electron spin resonance (ESR), and colorimetric detections. Direct measurement with ESR at physiologic conditions is difficult, if not impossible. Spin trapping techniques do not readily lend themselves to the study of intact organ. Colorimetric methods are less sensitive and specific (16,17).

The LSC system has a mechanical deficiency in measuring the fast reactions due to discrete sample system (drop of the vial to the detector) causing delay. Another disadvantage of LSC is the poor temperature control of the system. The newer luminometers are a better choice for assay because they are more efficient at counting photons and they possess a high degree of temperature regulation. In addition they can be linked to computers for data handling. In order to minimize the disadvantages efficient data calculations are established (18). Data expressions as maximum peak, time to maximum peak are available, but the most valid expression is the area under the curve (AUC). The curve is obtained by several counts taken with same time interval for a certain period. The calculation that have been found most useful for the AUC is the integration of the curve by the trapezoidal rule (a linear approximation). Although more exact methods are available, the accuracy of this method was proven to be sufficient(5).

In our laboratory, we have used chemiluminescence technique for the quantitation of ROS production in many tissue models (19-25), seminal plasma (26), and erythrocytes (27). Here we report a recent application where chemiluminescent detection of ROS was used in an acute pancreatitis model (28).

CHEMILUMINESCENT DETECTION OF ROS IN AN ACUTE PANCREATITIS MODEL: PROTECTIVE EFFECT OF COMBINED VITAMIN E AND C THERAPY

Acute pancreatitis remains as an important surgical problem with considerable morbidity and mortality. Its treatment is difficult and pathogenesis poorly understood. Various studies on the role of free radicals in the pathogenesis of acute pancreatitis were previously reported (30-31). Sanfey et al. (29) formed acute pancreatitis in *ex vivo* perfused canine pancreas by oleic acid infusion, partial duct obstruction, and an ischemia period of 2 hours. It was shown that enhanced generation of oxygen radicals added considerably to the damage observed in all three forms of pancreatitis. Guice et al. (30) showed that pretreatment with superoxide dismutase (SOD) and catalase reduced pancreatic damage in an *in vivo* cerulein induced pancreatitis model. These

results were recently confirmed by Wisner et al. (31). They showed that polyethylene-glycol-linked SOD minimized tissue edema and histological damage in cerulein-induced pancreatitis. All of these studies depend on beneficial effects of free radical scavengers or increase in lipid peroxidation products for stating the importance of free radicals etiopathologically. However, studies on direct detection of produced ROS in an acute pancreatitis injury model is lacking.

We have divided thirty female Wistar albino rats weighing 200-240 g into three groups. First group had sham operation. Experimental acute pancreatitis was performed by ligating the common bile duct at its point of entry into the duodenum (32) in the second and third groups. The third group received (vitamin E 100 IU/kg im) and vitamin C (10 mg iv) immediatiely and 12 hours after the operation. Twenty-four hours after the operation the animals were sacrified. Blood samples were taken for amylase, lipase, alkaline phosphatase, AST, and ALT determination, and pancreatic samples were used for pathological examination and chemiluminescence measurements. Chemiluminescence measurements were done using a liquid scintillation counter (Tricarb 1500; Packard Instruments, IL, USA) in out-of-coincidence mode with a single active photomultiplier tube. Fresh pancreatic samples were gently transferred to glass scintillation vials. Luminescence was recorded at room temperature after the addition of 0.2 mM lucigenin or 0.2 mM luminol. Counts were obtained at one minute intervals and the results were expressed as area under curve (AUC) for a counting period of 60 minutes corrected for tissue weight (cpm/mg tissue).

The significance of differences between experimental groups were estimated by one-way analysis of variance with Tukey-Kramer Multiple Comparison post-test. The differences were considered significant when the probability was $p<0.05$.

There have been various studies on the role of free radicals in the pathogenesis of acute pancreatitis. Moreover, in some clinical studies on patients suffering from pancreatitis, lipid peroxidation products seem to be important in the development of the disease (33). However, all of these studies were done by indirect estimations. In our study, acute pancreatitis induction with common bile duct ligation led to increase in luminol and lucigenin enhanced species detected by chemiluminescence (Table I).

Over the past decade, the ability of several agents to protect against pancreatitis has been evaluated (30-33). After detecting the enhanced free radicals in acute pancreatitis, we have tested the effect of antioxidants in therapy. Vitamin E is the major natural antioxidant existing in the lipid fraction and is known to be a potent chain-breaking antioxidant which prevents peroxidative damage in the tissues. Vitamin C is not only necessary for regeneration of vitamin E after its intereaction with free radicals but is also known to alleviate phagocyte mediated inflammatory response (34). In our study combined vitamin E and C therapy administered after pancreatitis induction significantly decreased ROS formation (Table I). Therefore, increased ROS were detected in acute pancreatitis and their etiopathological importance were also proven by the therapeutic efficiency of antioxidants. These results show that treatment with free radical scavengers continues to be beneficial even after the initial onset of free radical generation and subsequent damage. This has potential application in the clinical situation where the patient is admitted to the hospital after suffering from the first symptoms of acute pancreatitis.

Histopathologically, the panreatic tissue from the sham group were normal, but a grave form of pancreatitis characterised by infiltraton of neutrophils, necrotising vasculitis and multifocal hemorrhages were observed in the second group. There were only slight hemorrhages in the vitamin E and C treated group. Additionally, serum enzyme concentrations were significantly elevated in the pancreatitis group, and decreased in combined vitamin E and C treated group (Table II).

Chemiluminescence has been shown to be valuable for monitoring ROS formation in *in vivo* and *ex vivo*

Table I. Luminol and lucigenin enhanced chemiluminescence in a rat acute pancreatitis model
The values were given as mean ± SEM

	Luminol enhanced chemiluminescence	Lucigenin enhanced chemiluminescence
Sham group (n=10)	7186.3±1354.1	35077±7089
Pancreatitis group (n=10)	108295.6±30126*	21669.1±50945*
Vit E and Vit C treatment group (n=10)	24553.3±4644.4**	45172.9±8533.8**

*p<0.001 when compared to sham group
**p<0.01 when compared to pancreatitis group

systems. It further differentiates and quantitates the radicals separately, with the use of several enhancers of different selectivity. Lastly, CL has been shown to be a very sensitive assay system for determining the oxidative effects of ROS on different models.

Table II. Serum enzyme concentrations in a rat acute pancreatitis model
The values were given as mean ± SEM

	Amylase (IU/L)	Lipase (IU/L)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)
Sham group (n=10)	6292	4.3	245	76	143
Pancreatitis group (n=10)	24157*	558*	724*	340*	970*
Vit E and Vit C treatment group (n=10)	14182**	380**	422**	144**	554**

* $p<0.001$ when compared to sham group
** $p<0.01$ when compared to pancreatitis group

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