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Invited Review

The visualization of oxidant stress in tissues and isolated cells

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Summary. Many studies have implicated the role of oxidant stress in a wide range of human diseases and have led to the rapid expansion of research in this area. With many experimental approaches a direct detection of the production of reactive oxygen species (ROS) and free radicals is not possible. Free radicals are very reactive, short-lived and react in a non-specific way, so that ongoing oxidative damage is generally analyzed by measurement of secondary products e.g. H_2O_2, "oxidized" proteins, peroxidized lipids and their break-down products, "oxidized" DNA or by fluorographic analysis in combination with fluorescent dyes e.g. dichlorofluorescin (DCFH). The histochemical visualization of selected molecular markers for oxidative phenomena can often provide valuable information concerning the distribution of oxidative processes *in vivo*. A number of biochemical methods are available for the monitoring of almost all oxidant stress-related processes, although their applicability *in vivo* is limited.

This review summarizes the biochemical methods currently available for histochemical detection and indirect visualization of an excess of free radicals and ROS. The cited methods are discussed and the results obtained from their application are critically evaluated.

Key words: Oxidant stress, Histochemical detection, Marker, ROS

Introduction

The comprehensive term "oxidant stress" is currently employed to indicate a complex series of biochemical processes, which can affect living matter under several distinct conditions and produce both physiological and pathological effects. Oxidant stress can be loosely defined as the prevalence within the living cell of oxidizing species over the cellular antioxidant defences, and it is this 'pathological' perspective that has received the most attention over the past decades. Until recently, a considerable amount of research has in fact focused on the role of oxidant stress in mediating the cell damaging effects of a wide range of prooxidant agents (chemicals, drugs, pollutants); several detailed reviews have been published, dealing with the mechanistic aspects of these cytotoxic processes (Comporti, 1989; Farber et al., 1990; Janssen et al., 1993; Rubbo et al., 1996; Wiseman and Halliwell, 1996; Pompella, 1997).

At the same time, a number of studies in recent years have highlighted the role of oxidation/reduction (redox) reactions in the regulation of molecular mechanisms involved in cellular signal transduction (Powis et al., 1995; Lander, 1997; Nakamura et al., 1997; Sen, 1998). It is becoming clear that low "physiological" levels of oxidant agents (and reactive oxygen species in particular) can have physiological roles within cells; thus, the term "oxidant-mediated regulation" has recently been proposed as a more accurate alternative to "oxidant stress" (Cotgreave and Gerdes, 1998). This novel perspective is particularly intriguing, as it will surely advance our understanding of the role played by redox processes in human disease; oxidant stress has in fact been recognized as a primary factor in the pathogenesis of a number of notable human pathologies, such as atherosclerosis, ischemia-reperfusion, cancer or Alzheimer's disease (reviewed by Halliwell and Gutteridge, 1989).

Sophisticated and sensitive biochemical procedures are available for the determination of even minimal levels of oxidant stress *in vivo* (Packer, 1994). However, such approaches do not generally allow collection of information concerning the distribution of such phenomena *in situ*, even though this aspect is of great potential importance for the understanding of oxidative processes especially in the case of tissues with a heterogeneous cell composition, such as brain, lung or kidney. As a result, some laboratories - including our own - have approached the issue of determination of oxidant stress *in vivo* from a histochemical point of view. The possibility of developing specific and sensitive procedures able to directly reveal some of the biochemical changes induced by oxidant stress have

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therefore made feasible the discrimination of areas, cellular types and – possibly – subcellular sites which are involved in this phenomenon. This review will present an updated survey of the available histochemical approaches, of the problems encountered with some of them, and of the most relevant applications published in the literature.

Visualization of sites of production of reactive oxygen species (ROS)

ROS (superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen) are continuously produced during normal cellular metabolism at several different sites, e.g. mitochondrial respiratory chain, cytochromes P450 and b5, xanthine oxidase, and the NADPH-oxidase system of phagocytic cells (Halliwell and Gutteridge, 1989). Under several pathophysiological conditions, this 'basal' production of ROS can be considerably increased (inflammatory disease, ischemia-reperfusion). ROS are also produced by the action of a number of xenobiotics (Comporti, 1989) and 'redox-cycling compounds' (Kappus and Sies, 1981), including several drugs and transition metal ions, in particular iron. Figure 1 presents a simplified outline of the main sources of ROS and of their interconversions and interactions with radical nitrogen species.

The specific detection of superoxide anions in biological samples is made difficult by several methodological problems (Fridovich, 1997) and therefore requires the most accurate application of specificity controls. For many years, especially in studies using activated phagocytes, the reduction of nitroblue tetrazolium (NBT) to insoluble blue formazan has been widely used to evaluate superoxide production at light microscope level, as well as with electron microscopy (Hirai et al., 1992). However, the cytochemical visualization of subcellular sites of ROS production has predominantly been made possible by the outstanding contributions of M.J. Karnowsky and his coworkers, who defined and optimized the procedures for detection of hydrogen peroxide (by the cerium chloride method) and superoxide (by the Mn^{2+}-diaminobenzidine method), additionally providing insights into the cellular production of singlet oxygen (reviewed by Karnowsky, 1994). Using activated neutrophils, the cerium chloride method has also recently been used for detection of cellular sites of H_2O_2 production with the laser-scanning confocal reflectance microscope (Robinson and Batten, 1990). In addition to experiments with phagocytes, the same methods have successfully been employed for the demonstration of the

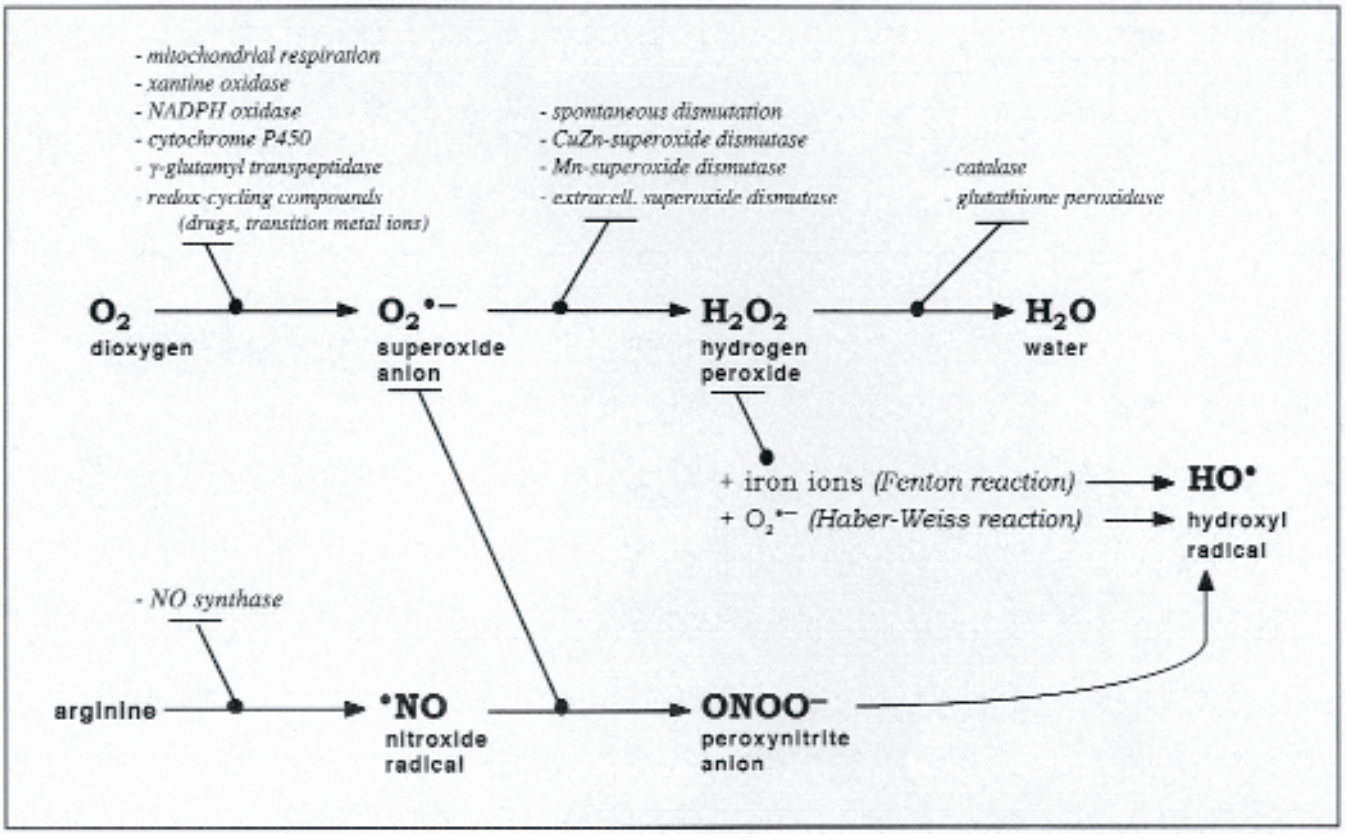

Fig. 1. Main processes involved in formation of reactive oxygen species, their interconversions and their interactions with nitrogen-centered reactive species.

production of ROS at the endothelial surface of cardiac vessels, during the first moments of reperfusion following a period of anoxia (Schlafer et al., 1990; Babbs et al., 1991).

Studies aimed at revealing the production of ROS are also currently being carried out using a method involving the preloading of living cells with 2',7'-dichlorofluorescin diacetate (DCF-DA), a compound whose fluorescence sharply increases in the presence of superoxide and other oxygen radical species (Fig. 2). DCF-DA has thus been successfully employed in the investigation of whole organs and isolated cells, as well as in flow cytometry applications (Royall and Ischiropoulos, 1993; Tsuchiya et al., 1994). Even so, the actual specificity of DCF-DA in detecting individual ROS has never been elucidated with sufficient detail; one interesting investigation in this direction has indeed questioned the ability of DCF-DA to detect superoxide anion, and has shown that DCF-DA can itself serve as a substrate for xanthine oxidase and other cellular peroxidases (Zhu et al., 1994).

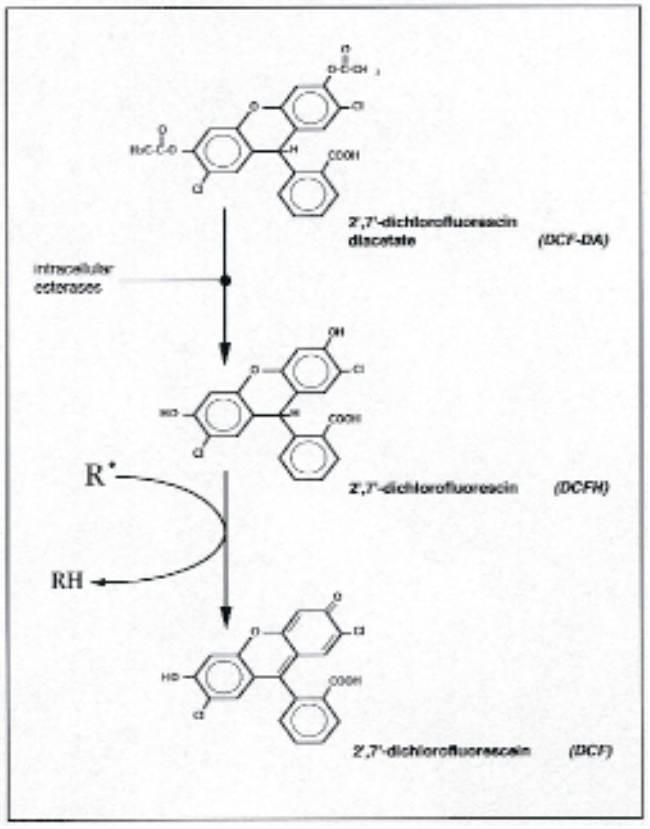

Fig. 2. Detection of oxidants within living cells by means of 2',7'-dichlorofluorescin diacetate (DCF-DA). Once DCF-DA has penetrated into the cell, cytosolic esterases will promptly release the non-cell permeable compound, DCFH, which is thus trapped in the cell. The observation of the oxidation of DCFH to the highly fluorescent derivative DCF is interpreted as the visible effect of oxidant conditions taking place intracellularly.

Visualization of aldehydes and carbonyls derived from the peroxidation of unsaturated lipids

Under selected circumstances, the progression of oxidant stress within a cell can lead to the initiation of a highly destructive, self-propagated chain of oxidative reactions involving cell membrane lipids, a process termed lipid peroxidation (LPO). This process ultimately results in the oxidative fragmentation of polyunsaturated fatty acids of cellular phospholipids, and includes a complex series of biochemical steps which have been described in detail by several authors (Horton and Fairhurst, 1987; Halliwell, 1990; Esterbauer et al., 1991). With respect to histochemistry, the most prominent aspect of LPO is the formation of a wide range of aldehyde and carbonyl compounds following the breakdown of polyunsaturated fatty acids; a comprehensive description of such products has been provided by Esterbauer et al. (1989). Of these LPO-derived products, the lipophilic ones will remain associated with the lipid phase, while others (e.g. malonaldehyde, MDA, one of the most abundant LPO products) will promptly diffuse into aqueous media. An important class of LPO products is the α,ß-unsaturated aldehydes, which show variable degrees of reactivity with amino acid residues in protein. Altogether - as shown schematically in Figure 3 - the process of LPO will result in a marked increase in the amount of carbonyl and aldehyde groups in cellular lipids and proteins, along with a decrease in protein reduced thiol groups. Thus, the occurrence of LPO in a given specimen can be inferred by the histochemical identification of these biochemical alterations.

The direct Schiff reaction has long been employed for the identification of aldehydes in tissues, even though the exact mechanism for the generation of chromogen has never been convincingly elucidated, despite a long-lasting debate (see e.g. Hörmann et al., 1958). With respect to LPO-derived aldehydes, Schiff's reaction was first used for the visualization of areas with decreased sensitivity to induction of lipid peroxidation *in vitro* in cryostat sections obtained from the liver of rats following administration of a carcinogen (Benedetti et al., 1984). Subsequently, the same procedure was applied to the detection of lipid peroxidation in vivo in the whole animal (Pompella et al., 1987), using a model involving intoxication with bromobenzene, a glutathione-depleting agent with a strong prooxidant action on several rat organs (Comporti, 1987). Figure 4 shows the distribution of LPO in mouse liver, as assessed by the direct Schiff reaction, after treatment with bromobenzene and with allyl alcohol, another distinct glutathione-depleting prooxidant.

The direct Schiff reaction was subsequently employed with success under other experimental conditions, allowing the demonstration of the selective involvement of rat *substantia nigra* during *in vitro* iron-induced lipid peroxidation (Tanaka et al., 1992), and of rat tubular proximal epithelium during *in vivo* lipid

peroxidation induced by the nephrocarcinogen iron nitrilotriacetate (Toyokuni et al., 1990). Very interesting results were obtained by Masuda and Yamamori, who studied the distribution of LPO in relation to that of cell injury in rat livers subjected to anterograde *vs*. retrograde perfusion with different prooxidant toxins and under different oxygen tensions (Masuda and Yamamori, 1991a,b).

However, the use of Schiff reaction is limited by a somewhat poor reproducibility, and the strong acidity of the reagent can induce false positivite results in tissues rich in plasmalogens, such as myocardium, where the so-called "pseudoplasmal" reaction can be seen (reviewed in Pearse, 1985). These difficulties have therefore warranted the development of alternative procedures. Good results have been obtained using a reaction based on 3-hydroxy-2-naphthoic acid hydrazide (NAH) followed by coupling with a tetrazolium salt (Fig. 5); the reliability of the NAH reaction has been assessed by means of microspectrophotometrical analysis of tissue sections and comparison with data obtained by biochemical determination of LPO in the same specimens (Pompella and Comporti, 1991). The use of the NAH reaction allowed visualization of regions first affected by lipid peroxidation *in vivo* following the intoxication with haloalkanes (carbon tetrachloride, bromotrichloromethane); such LPO levels had proven to be lower than the detection limit possible with the direct Schiff reaction.

A further improvement in the histochemistry of lipid peroxidation was obtained by the employment of fluorescent reagents for the identification of LPO-derived carbonyls in tissues and isolated cells. Fluorochromes have in fact enabled an appreciable increase in the sensitivity of detection together with the possibility of analysis by means of confocal laser scanning fluorescence microscopy with image video-analysis. Interesting results with this procedure have been obtained by exploiting the fluorescence of the NAH reagent itself (Pompella and Comporti, 1993). An example of results obtainable with this procedure is shown in Figure 6, where the selective involvement of the first rows of pericentral hepatocytes by lipid peroxidation induced *in vivo* in the rat by intoxication with carbon tetrachloride can be seen. An alternative approach to fluorescent derivativization of cellular

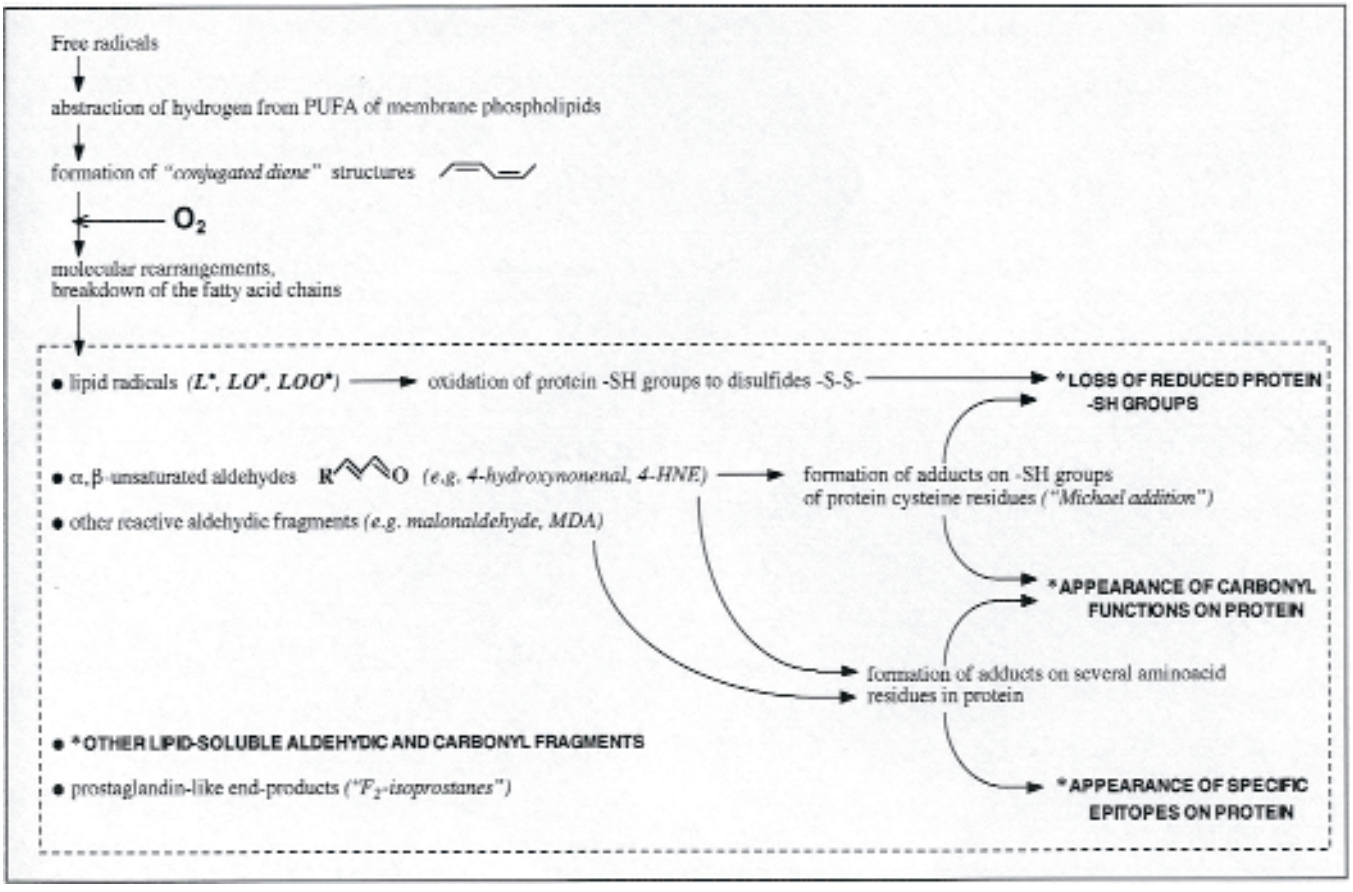

Fig. 3. Main products of lipid peroxidation and their modifying effects on proteins. Several classes of compounds are originated during the process of lipid peroxidation, some of which are directly detectable by histochemical procedures and/or lead to histochemically-detectable alterations in proteins (indicated with an asterisk).

carbonyls together with confocal laser scanning microscopy was followed by others, using a biotin-labelled hydrazide coupled with fluorescent-conjugated streptavidin (Harris et al., 1994).

An additional tool for the detection of LPO is the naturally fluorescent fatty acid, *cis*-parinaric acid. Once pre-loaded in living cells, *cis*-parinaric acid is readily consumed during lipid peroxidation, thus allowing the monitoring of the lipid peroxidation process in the form of a fluorescence decrease. To date, the procedure has found successful application in flow cytometry (Hedley and Chow, 1992).

Visualization of oxidative changes in cellular protein ('protein oxidation')

The oxidative modification of proteins by reactive oxygen species and other reactive compounds has been recognized to play a role in the progression of several pathophysiological processes, including a range of notable diseases and aging (Davies and Dean, 1997).

As comprehensively reviewed by Stadtman and Barlett (1997), there are three major pathways leading to protein oxidation: i) the so-called metal-catalyzed oxidation of amino acid residues; ii) lipid peroxidation; and iii) protein glycation and glycoxidation reactions (Fig. 7). In the case of metal-catalyzed protein oxidation, the direct oxidation of amino acid residues is the consequence of interaction with the polypeptide chain of hydroxyl radicals, generated e.g. by ionizing radiation, or by the Fenton reaction between H_2O_2 and iron ions (Fig. 1). Almost all amino acid residues can be involved, and the process often results in peptide bond cleavage and protein-protein cross link formation.

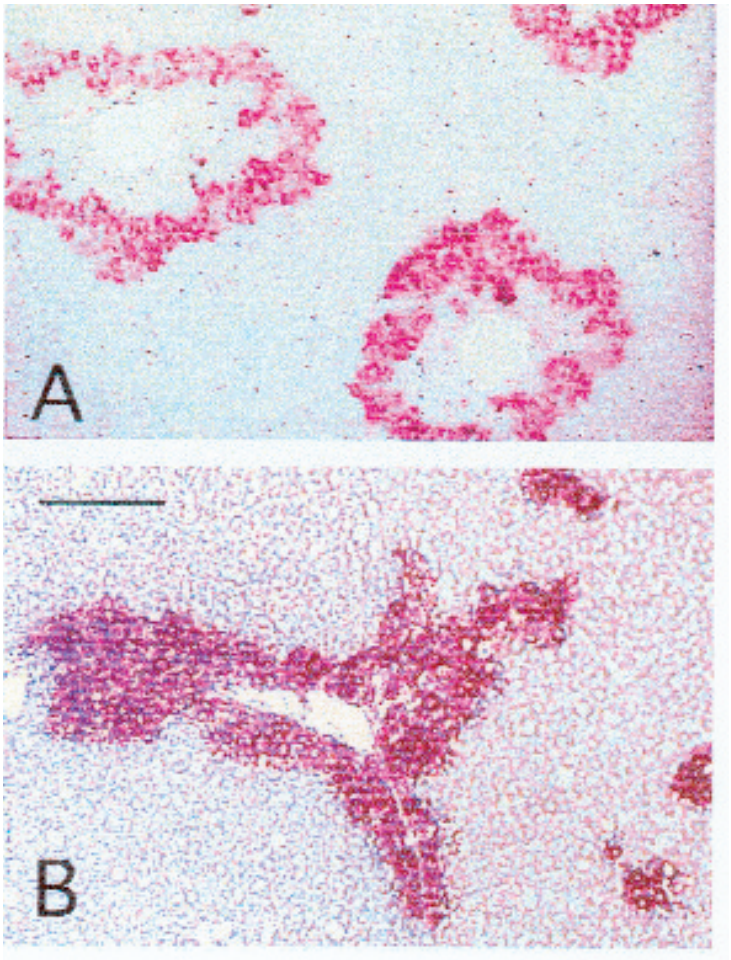

Fig. 4. Visualization by direct Schiff reaction of tissue areas becoming involved by lipid peroxidation *in vivo* in mouse liver, following the administration of glutathione-depleting toxins. **A.** Medio-lobular distribution of LPO (purple stain) caused by bromobenzene intoxication. **B.** Strict periportal localization of LPO induced by intoxication with allyl alcohol. See Pompella et al. (1987) for details of the procedures employed. Bars: 50 μm.

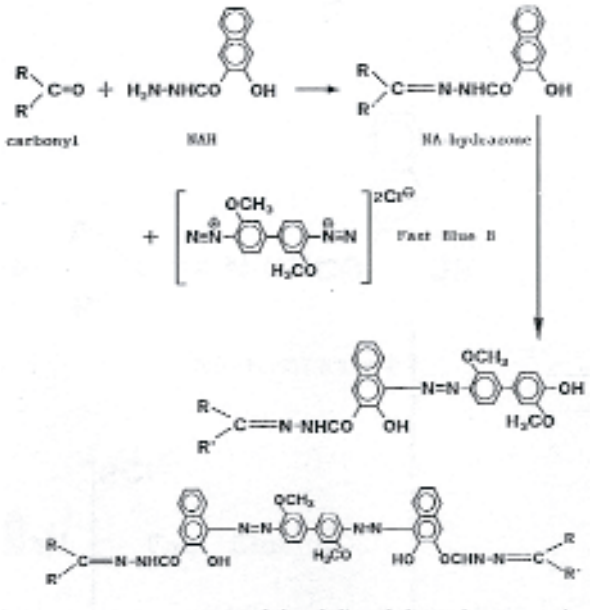

Fig. 5. The naphthoic acid hydrazide-fast blue B (NAH-FBB) reaction or detection of carbonyl groups in tissues (Pompella and Comporti, 1991). Carbonyls are first converted to naphthoic hydrazones by reaction with NAH; the coupling with the diazonium salt yields then violet-coloured azo dyes.

A second pathway leading to protein oxidation is that of lipid peroxidation. As discussed in the previous section, the involvement of proteins in reactions with LPO-derived reactive compounds can in fact result in an increase in protein-associated carbonyl functions, along with a decrease in detectable reduced protein thiols (Fig. 3). This is the result of a direct addition of α,ß-unsaturated aldehydes to amino acid residues in protein, which can lead to the formation of adducts in which the aldehyde grouping remains available for further reactions (Fig. 8). Malonaldehyde, which is one of the most abundant LPO aldehydic products, has also been shown to introduce carbonyl functions in protein (Burcham and Kuhan, 1996).

Glycation (i.e. the binding of glucose to protein) is commonly observed in diabetes mellitus patients, and can facilitate protein oxidation. Combined glycation and oxidation, 'glycoxidation', occurs when oxidative reactions affect the initial products of glycation, and results in irreversible structural alterations in proteins; this eventually leads to the accumulation of the so-called 'advanced glycation end-products' (AGEs) in tissue (Vlassara et al., 1994).

For the detection of protein-associated carbonyl functions, the method originally developed by Levine et al. (1994) has recently been adapted in our laboratory for histochemical application (Pompella et al., 1996a,b). As demonstrated in Figure 9, the procedure consists of a first step, in which protein carbonyls are derivatized by 2,4-dinitrophenyl hydrazine (2,4-DNPH) to yield the corresponding 2,4-dinitrophenyl hydrazones. In a second step, the dinitrophenyl (DNP) groups which become associated with proteins in this way are detected immunochemically by means of a commercial anti-DNP antiserum; finally, antibodies bound to specimens are identified with a conventional biotin-avidin system, or equivalent (Pompella et al., 1996a).

In principle, the 2,4-DNPH/anti-DNP procedure should reveal all kinds of carbonyls becoming associated with protein, irrespective of their origin. With this method, oxidized proteins have been visualized in several interesting studies, e.g. in activated neutrophil

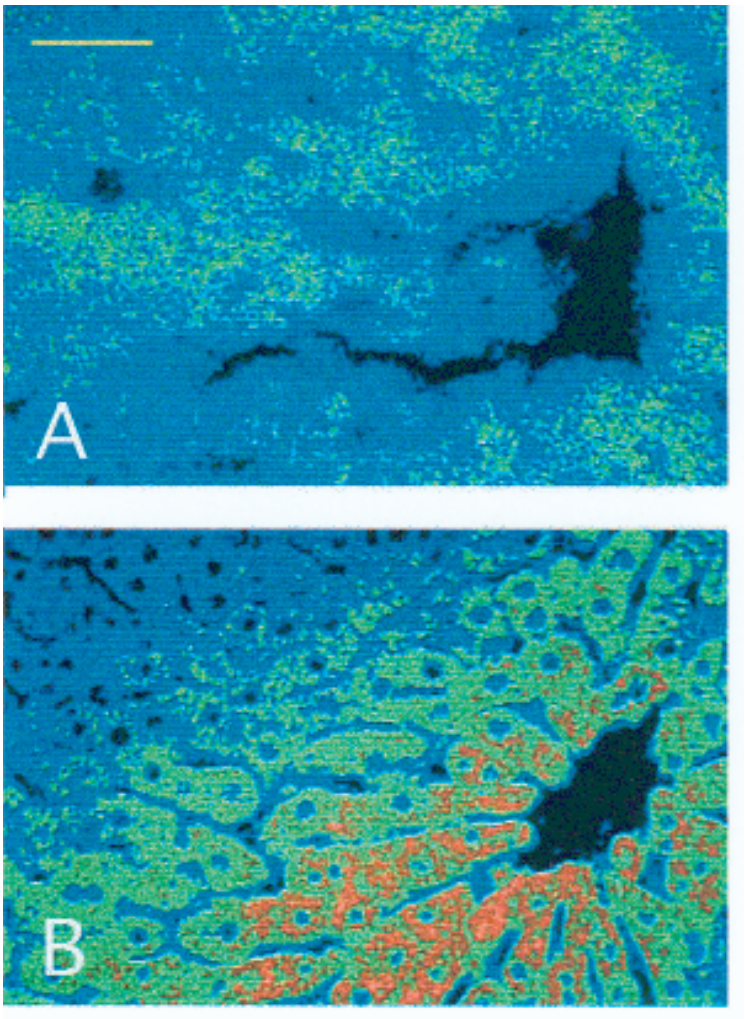

Fig. 6. Confocal laser scanning fluorescence imaging of lipid peroxidation in rat liver in vivo, following fluorescent derivativization of cellular carbonyls with NAH. Fluorescence intensities are translated into false-color images generated by the computer, according to a scale going from blue (lower intensities) to green, yellow (middle intensities) and red (higher intensities). **A.** Control rat liver. **B.** Rat liver following intoxication with the prooxidant haloalkane carbon tetrachloride. See Pompella and Comporti (1993) for details of the procedures employed. Bar: 50 μm.

phagocytes (Pompella et al., 1996a; Cambiaggi et al., 1997), in brain tissue from Alzheimer patients (Smith et al., 1996) and in sarcoma cells exposed to prooxidant treatments (Fig. 10) (Frank et al., 1998). However, the usefulness of the 2,4-DNPH/anti-DNP procedure for the histochemical detection of protein glycoxidation products has not yet been evaluated.

Another important parameter which can be used as a marker of oxidant insult to protein is the loss of reduced -SH groups, which has mainly been investigated with reference to toxic cell injury and lipid peroxidation (Bellomo and Orrenius, 1985; Pompella et al., 1991). Modern histochemistry of protein thiols was developed and optimized by G. Nöhammer, who laid down the foundations for semi-quantitative microspectrophotometry of protein reduced thiols, protein disulfides and protein mixed disulfides (Nöhammer, 1981, 1982). These procedures have found interesting applications in the histochemical evaluation of protein thiol redox status in neoplastic and preneoplastic cells (Nöhammer et al., 1986; Pompella et al., 1996b). Fluorescent labelling procedures have recently been developed for the visualization of both total and cell surface protein reduced thiols at the single cell level, by laser-scanning confocal microscopy (Pompella et al., 1996a) (Fig. 11).

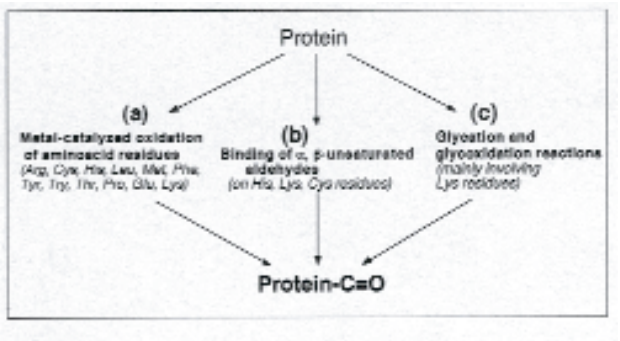

Fig. 7. Three distinct pathways to protein oxidation (= increase of carbonyl groups in proteins). Carbonyl groups are introduced into proteins **(a)** as a result of direct oxidant attack to protein, through the metal-catalyzed oxidation of side chains of several amino acids, **(b)** following a process of lipid peroxidation, by the reaction of the double bond of alpha, beta-unsaturated aldehydes (e.g., 4-HNE) with amino, sulfhydryl and imidazole groups in protein, and **(c)** by reaction of protein amino groups with carbohydrates, through glycation and glycoxidation reactions.

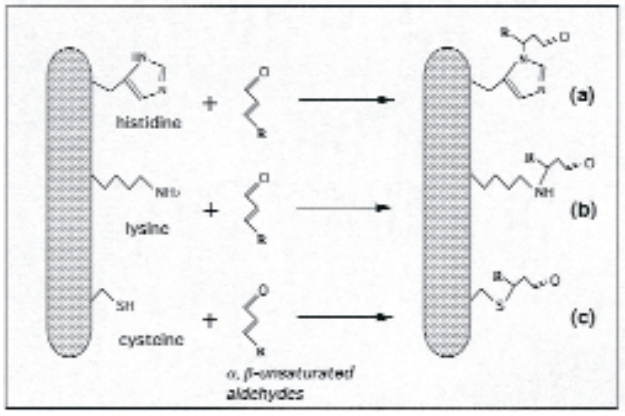

Fig. 8. Protein carbonyl adducts resulting from the reaction of selected amino acid side chains with LPO-derived alpha, beta-unsaturated aldehydes. The imidazole moiety of histidine residues, the epsilon-amino group of lysine residues, and the sulfhydryl group of cysteine residues, may all undergo addition reactions with the alpha, beta-double bond of alpha, beta-unsaturated aldehydes (e.g., 4-HNE), to form **(a)** the corresponding tertiary amine derivatives, **(b)** the secondary amine, and **(c)** thioester structures.

Immunodetection of oxidant stress-induced epitopes in proteins and nucleic acids

The immuno-histochemical approach to oxidant stress has expanded rapidly over the past few years, and today represents the tool of choice for the specific detection of oxidative changes in tissue and cells. Following the structural alterations introduced by an oxidant insult, proteins can in fact easily acquire new antigenic properties due to the appearance of new specific epitopes on the polypeptide chain. This is primarily the case with reactive aldehydes derived from lipid peroxidation, which are able to bind to several amino acid residues, as outlined in Figure 3. By means of specific polyclonal or monoclonal antibodies, the occurrence of malonaldehyde (MDA) and 4-hydroxy-nonenal (4-HNE) bound to cellular protein has thus been

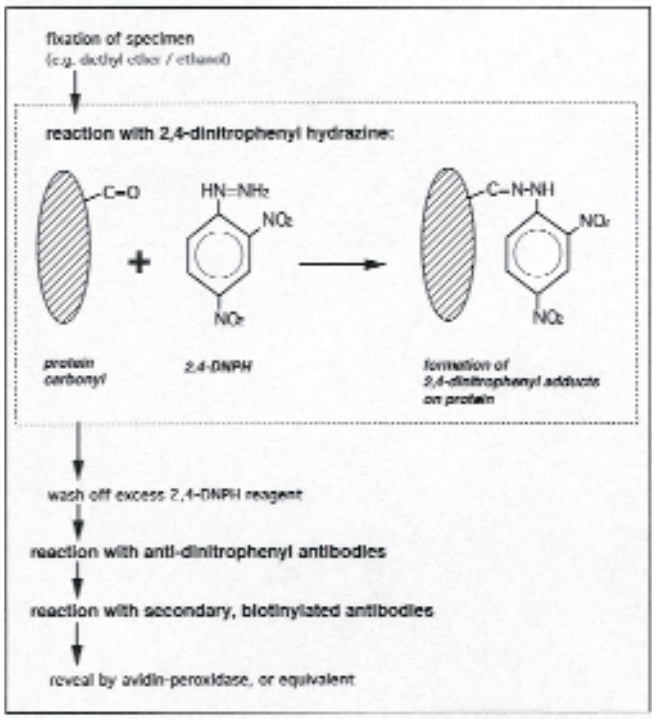

Fig. 9. The two-step procedure for the histochemical determination of protein carbonyl groups. See Pompella et al. (1996a) for details.

documented under a number of experimental and clinical conditions. Lipid peroxidation has been demonstrated in this way in collagen-producing fibroblasts (Chojkier et al., 1989; Bedossa et al., 1994), in the liver of human alcoholics (Niemelä et al., 1994), hepatitis C patients and other chronic liver diseases (Paradis et al., 1997a,b), in the arterial wall during experimentally-induced atherosclerosis (Palinski et al., 1989), in activated neutrophils (Quinn et al., 1995; Cambiaggi et al., 1997), in nigral neurons of Parkinson patients (Yoritaka et al., 1996), in ferric-nitrilotriacetate-induced renal carcinogenesis (Uchida et al., 1995) as well as in human renal carcinoma (Okamoto et al., 1994). Most of these studies were largely made possible by the availability of thoroughly characterized monoclonal antibodies (Toyokuni et al., 1995; Waeg et al., 1996).

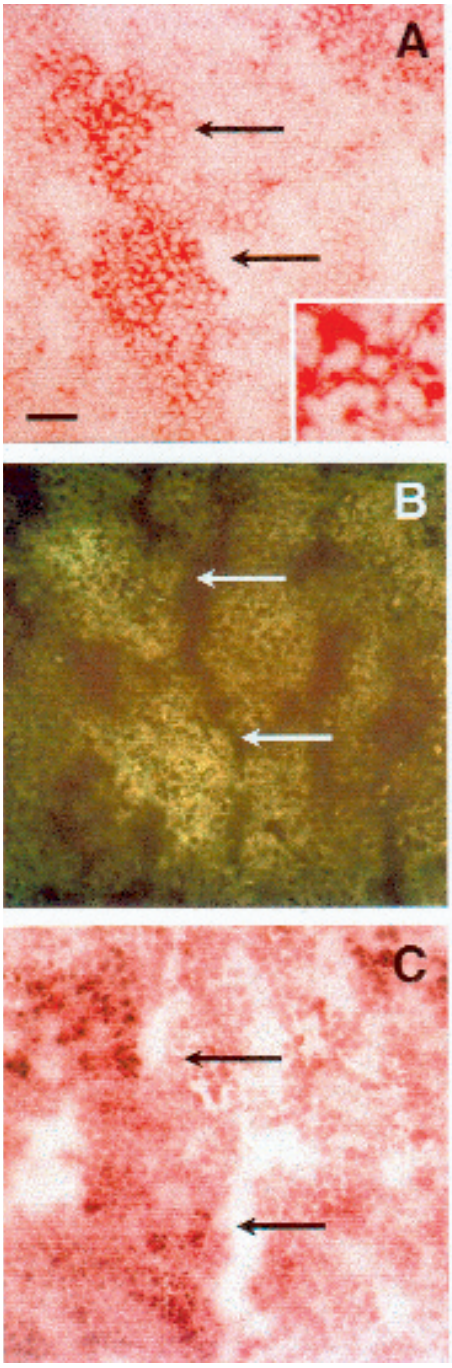

Fig. 10. Colocalization of protein oxidation (**A**, DNPH/anti-DNP reaction) and lipid peroxidation. (**B**, immuno-fluorescence by polyclonal antiserum against protein-4-HNE adducts) at sites of cellular injury, (**C**, hematoxylin/eosin). Rat DS-sarcoma cells treated in vivo with hyperthermia plus respiratory hyperoxia and xanthine oxidase. Bar: 50 μm. Reproduced from Frank et al. (1998).

Specific epitopes are also present in oxidized low-density lipoproteins (ox-LDL), a distinctive class of oxidized proteins probably involved in the pathogenesis of atherosclerosis. The exact nature of such epitopes is a matter of debate, although is seems certain that the antigenicity of oxLDL can be at least partially accounted for by the binding of LPO-derived aldehydes, such as MDA and 4-HNE, to the LDL apoprotein moiety (Chen et al., 1992; O'Brien et al., 1996; Requena et al., 1997). By means of polyclonal and monoclonal antibodies raised against in vitro-oxidized LDL, the immunohistochemical visualization of ox-LDL has been repeatedly reported in atherosclerotic lesions (Palinski et al., 1989; Ylä-Herttuala et al., 1990; Paolicchi et al., 1999).

With respect to AGE-modified proteins, immunohistochemical studies aimed at determining the sites of accumulation of these products have generally employed antibodies specific for N-ε-(carboxymethyl) lysine, the main antigenic structure produced during protein glycoxidation (Ikeda et al., 1998). In this way, AGEs have recently been detected in several disease conditions (Matsuse et al., 1998; Sasaki et al., 1998; Sun et al., 1998; Takayama et al., 1998).

Besides protein oxidation, resulting in increased levels of protein carbonyls, a different type of oxidative attack on protein must also be considered, i.e. that caused by reactive nitrogen species (Fig. 12). The peroxynitrite anion $ONOO^-$ can in fact react with aromatic amino acids, probably through the formation of the nitronium cation NO_2^+ and of the $^\bullet NO_2$ radical. This will primarily result in the addition of nitrate groups to the ortho position of tyrosines, a process referred to as 'protein nitration' (Darley-Usmar et al., 1995). Histochemically, protein nitration can be documented by means of immunomethods employing anti-nitrotyrosine antibodies (Beckman et al., 1994; Good et al., 1996; Virág et al., 1998).

Finally, recent studies have explored the possibility of extending histochemical investigations to the detection of oxidized DNA. DNA damage by both reactive oxygen and reactive nitrogen species is in fact a prominent aspect of a number of oxidant stress conditions. Toyokuni and coworkers have developed a quantitative immunohistochemical procedure for determination of 8-hydroxy-2'-deoxyguanosine - one of the main new epitopes on oxidized DNA - by means of a specific monoclonal antibody (Toyokuni et al., 1997).

Final remarks

Through the development of numerous methods and

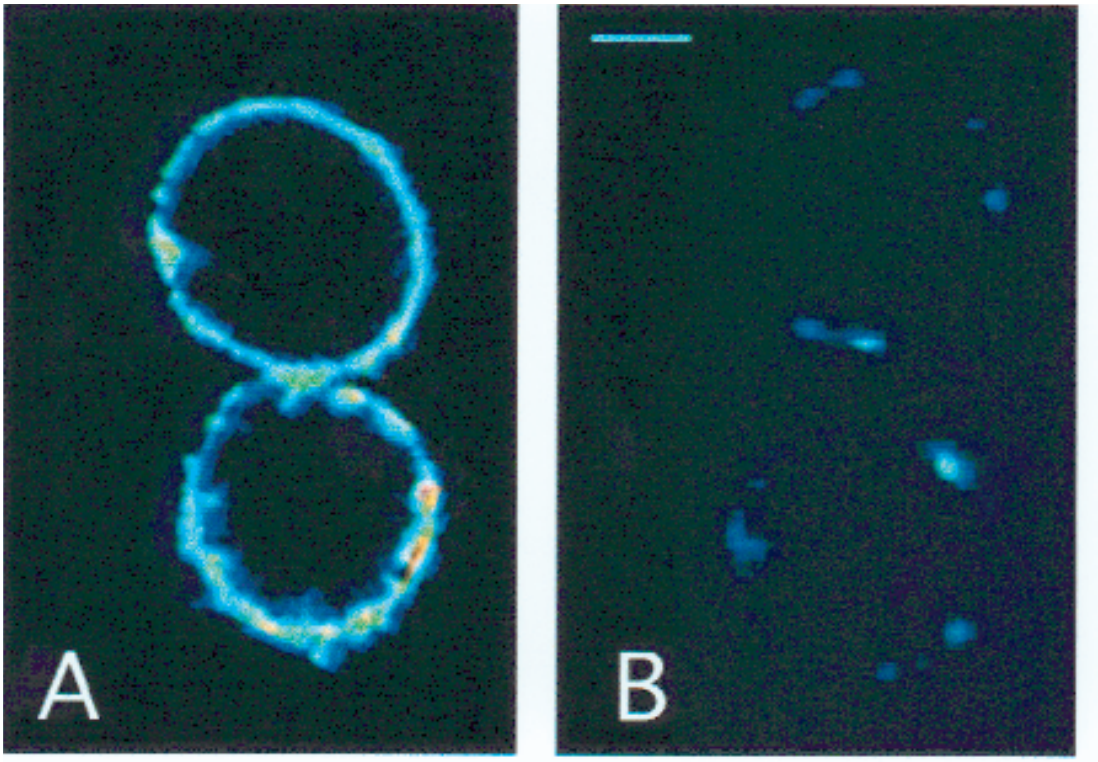

Fig. 11. Selective visualization of cell surface reduced protein sulfhydryl groups by MPB-based immunofluorescence. Confocal laser scanning fluorescence imaging; see Pompella et al. (1996a) for details of the procedures employed. **A.** Control HPBALL leukemia cells, showing a ring of reduced protein sulfhydryls at their surface. **B.** Loss of surface protein sulfhydryls following the exposure of cells to the lipid peroxidation product, 4-hydroxynonenal (4-HNE). Bar: 10 μm.

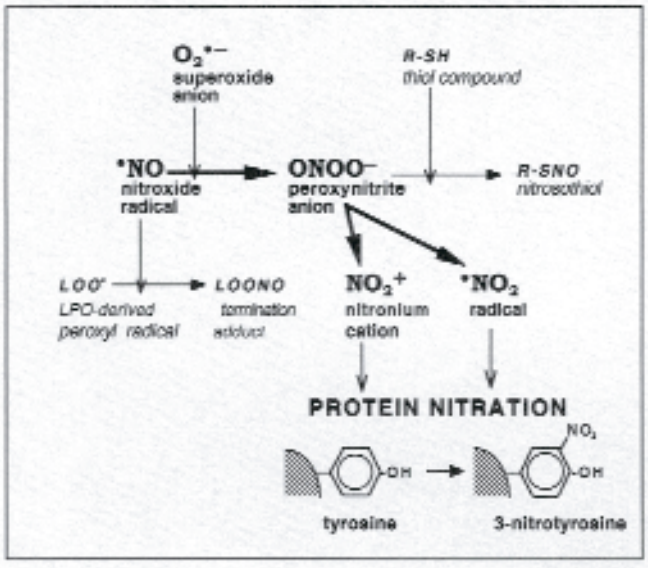

Fig. 12. Reactions of the reactive nitrogen species, nitroxide radical and peroxynitrite anion, leading to the formation of adducts on protein tyrosine residues ("protein nitration").

specific agents as outlined in this review, histochemistry has now become one of the most important experimental approaches available to researchers investigating oxidant stress. Although oxidative alterations have long been known to be associated with several disease conditions, the actual involvement of oxidant stress in the *pathogenesis* of a given condition has been convincingly documented only in a small number of cases to date. When compared to even the most sophisticated and specific biochemical determinations, the histochemical approach to evaluating oxidant stress offers the advantage of providing a 'certification' that this process is occurring at a given time and at a given site, either *in vitro* or *in vivo*. Information obtained in this way can often add significant details to an investigation of oxidative alterations in biological samples, thus assisting in the elucidation of the precise role of oxidant stress in experimental and clinical processes.

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