Detection of Protein Oxidation in Rat-1 Fibroblasts by Fluorescently Labeled Tyramine†

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Received December 8, 2000; Revised Manuscript Received April 13, 2001

ABSTRACT: Oxidative damage to proteins has been postulated as a major cause of various degenerative diseases including the loss of functional capacity during aging. A prominent target for oxidation by reactive oxygen species (ROS) is the tyrosine residue. Here we present a highly sensitive method for the detection of tyrosyl radical formation in cells. The method is based on the fluorescein-labeled tyrosine analogue, tyramine, which upon oxidation may couple to proteins carrying a tyrosyl radical. Coupling of the probe (denoted TyrFluo) to standard proteins could be induced by generating ROS with horseradish peroxidase/hydrogen peroxide, SIN-1 or with peroxides (cumene or hydrogen peroxide) in combination with a transition metal. TyrFluo added to rat-1 fibroblasts remained outside the cell, whereas the acetylated form (acetylTyrFluo) was membrane-permeable and accumulated in the cell. Exposure of the cells to oxidative stress in the presence of either TyrFluo or acetylTyrFluo gave a cellular labeling characteristic for each probe. Western blot analysis confirmed that each probe labeled a specific set of proteins. This new method for the detection of ROS-induced oxidation of proteins may mimic the tendency of oxidized proteins to form dityrosine bonds.

Although oxygen is essential for life, it can give rise to a variety of reactive oxygen species (ROS)[1] such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and superoxide ($O_2^{\cdot -}$), as part of normal metabolism or produced by irradiation or environmental factors. Under normal conditions, the body is able to eliminate these highly reactive compounds by means of enzymatic reduction, antioxidants (endogenous and exogenous), and metal chelators. When the level of ROS exceeds the defense mechanisms, a cell is in a state of oxidative stress. Under these circumstances, proteins may become irreversibly damaged. Proteins modified by oxidation have been shown to accumulate during aging (*1*−*5*), and as a result of several degenerative diseases including inflammatory diseases (*6*), atherosclerosis (*7, 8*), neurological disorders (*9*), ischemia and reperfusion injury, and carcinogenesis (*10*).

Whereas most amino acids can be oxidized, ROS-induced oxidation of, in particular, tyrosine and cysteine may have profound effects on cell function as these residues are often located in the active site of enzymes. Oxidation of essential tyrosine residues has been observed for superoxide dismutase (*11*) and glutathione reductase (*12*) resulting in their inactivation. Intermolecular cross-linking of enzymes involved in signal transduction by dityrosine formation has been detected in cells (*13, 14*).

Tyrosine oxidation may lead to the formation of tyrosyl radicals. These radicals are formed by reaction with hypochlorite, peroxynitrite, or by radicals formed in transition metal ion-catalyzed Fenton and Haber-Weiss reactions (e.g., H_2O_2/Fe^{2+}) (*15*−*17*). Peroxidases are also important sources of tyrosyl radicals. Protein tyrosyl radicals may form intra- or intermolecular *o*,*o*′-dityrosine bonds (*18*). It is known that the concentrations of *o*,*o*′-dityrosine bonds increase with aging in heart, skeletal muscle, and lens proteins (*19*, *20*). Another oxidation product is 3-nitrotyrosine that can be introduced into proteins as a result of tyrosine nitration by peroxynitrite, a product of superoxide ($O_2^{\cdot -}$) and nitric oxide (·NO) (*21*, *22*), by peroxidase-catalyzed oxidation of nitrite (*23*) and by nitric oxide reaction with protein tyrosyl radicals (*24*). Moreover, in human neutrophils the myeloperoxidase-hydrogen peroxide-chloride system oxidizes L-tyrosine yielding 3-chlorotyrosine (*25*) which is found in elevated amounts in low-density lipoprotein isolated from human atherosclerotic intima (*26*).

Despite the interest in tyrosine oxidation and its effect on protein function, the identity of the proteins that are major targets for ROS-induced tyrosyl radical formation remains to be resolved. Here we present a highly sensitive method

† This research was supported by Unilever, Vlaardingen, The Netherlands, and the Technology Foundation STW (Grant No. UBI 4443), Applied Science Division of NWO, and the Technology Program of the Ministry of Economic Affairs.

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[1] Abbreviations: ROS, reactive oxygen species; HRP, horseradish peroxidase; pre-nsL-TP, presequence of nonspecific lipid transfer protein; TyrFluo, tyramine linked to 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid; acetylTyrFluo, acetylated form of TyrFluo; SIN-1, 3-morpholinosydnonimine, hydrochloride; HBSS, Hanks' balanced salt solution; TBST, Tris-buffered saline containing 0.2% tween-20; PBS, phosphate-buffered saline.

10.1021/bi002795s CCC: $20.00
Published on Web 06/08/2001

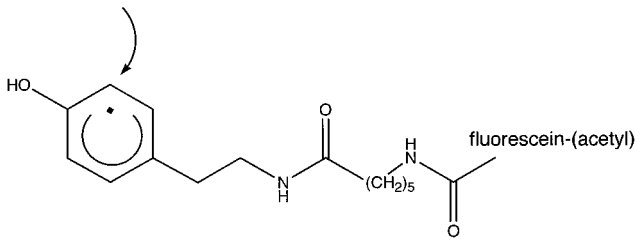

FIGURE 1: Tyramine−fluorescein conjugates (TyrFluo/acetylTyrFluo)• TyrFluo consists of tyramine linked to 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid. AcetylTyrFluo is the acetylated form of TyrFluo. The arrow indicates the site of oxidative attack by ROS. The radical may form an *o,o′*-dityrosine bond with tyrosyl radicals of proteins.

for the detection of dityrosine formation in intact cells. The method makes use of a fluorescein-labeled tyrosine analogue (i.e., tyramine) which upon oxidation by ROS is converted into a tyrosyl radical that can form cross-links with oxidized tyrosine residues in target proteins. As a result of this coupling reaction, these proteins become fluorescently labeled making them suitable for identification. We will present data to show that this probe can be used for the detection of oxidized proteins both inside and outside the cell depending on whether the acetylated or the nonacetylated form is used.

MATERIALS AND METHODS

Materials. 6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester, and SIN-1 (3-morpholinosydnonimine, hydrochloride) were purchased from Molecular Probes Europe (Leiden, The Netherlands). Tyramine and HBSS (Hanks' Balanced Salt Solution) were from Sigma-Aldrich (Bornem, Belgium). Anti-fluorescein (HRP-conjugated polyclonal antibody against fluorescein) was from Biogenesis (Poole, UK), SDS−PAGE low range molecular weight standards from Bio-Rad (Veenendaal, The Netherlands) and Protifar, a low-fat milk powder, from Nutricia (Zoetermeer, The Netherlands). Rat liver pre-nsL-TP expressed in *Escherichia coli* was purified according to ref *27*. The mounting solution Mowiol was from Hoechst (Frankfurt am Main, Germany).

Synthesis of the Tyramine−Fluorescein Conjugates. Both tyramine (1 mg) and the succinimidyl ester of 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid (1 mg) were dissolved in dimethylformamide (50 μL) and then added to 0.2 M bicine, pH 8.5 (0.2 mL). The coupling reaction was performed at room temp for 2 h. Synthesis of the conjugate was confirmed by thin-layer chromatography using an alkaline solvent (chloroform/methanol/25% ammonia/water = 90/54/5.5/5.5 v/v). Chloroform/methanol/25% ammonia (2/1/0.5 v/v) was added to the reaction mixture giving phase separation. The conjugate TyrFluo (Figure 1) was present in the water phase whereas free tyramine was dissolved in the chloroform phase. The water phase was removed by vacuum evaporation in a rotary evaporator. TyrFluo was taken up either in water or in tetrahydrofurane. In tetrahydrofurane, TyrFluo was acetylated by adding 4-(dimethylamino)-pyridine (0.5 mg) and acetic anhydride (3 μL) yielding acetylTyrFluo (Figure 1). As a result of acetylation, the solution became colorless. Ethanol was added to react with the excess of acetic anhydride. The concentrations of both conjugates were determined spectrophotometrically (ϵ_{495} = 73 000 M^{-1} cm^{-1}) in 1 M NaOH.

Labeling of Standard Proteins. SDS−PAGE low range molecular weight standards (6 μg/protein) were dissolved in PBS (pH 7.4). In the presence of TyrFluo (5 μM) the mixture was oxidized for 1 h at 37 °C by addition of CumOOH/hemin (2 mM), H_2O_2/Fe^{2+} (0.1 mM), SIN-1 or HRP/H_2O_2 (10 mM) in a final volume of 20 μL in the presence or absence of 500 μM tyramine. In some experiments, pre-nsL-TP was added to the standards. The reaction was stopped by adding 20 μL of sample buffer [100 mM Tris-HCl (pH 7.4), 100 mM DTT, 2% SDS, 20% (v/v) glycerol, bromophenol blue] and the proteins were denatured at 95 °C for 5 min.

Labeling of Proteins in Rat-1 Fibroblasts. Rat-1 fibroblasts were cultured to subconfluency (density of 80%) on plastic Petri dishes (150 mm^2) in Dulbecco's Modified Eagle Medium containing 7.5% (v/v) fetal calf serum. Cells were washed twice with Hanks' balanced salt solution (HBSS). The cells were preloaded with acetylTyrFluo (5 μM) in 5 mL HBSS/10mM Tris-HCl (pH 7.4) for 10 min at 37 °C and washed gently with HBSS. Then the cells were submitted to stress by adding 100 μM CumOOH/0.1 μM hemin or 100 μM H_2O_2 in 5 mL HBSS for 10 min at 37 °C so as to label the intracellular proteins. Under the same conditions, the cells were submitted to stress (0.2 mU HRP/1 μM H_2O_2) in the presence of TyrFluo (3 μM) so as to label extracellular proteins. At the end of the labeling cells were washed with PBS and prepared for fluorescence microscopy. Cells were fixed with 4% paraformaldehyde/0.1% Triton X-100 in PBS and washed four times with PBS to remove noncovalently bound probe. After the last wash with water, the fixed cells were mounted in Mowiol. For the analysis of labeled proteins by SDS−PAGE, sample buffer (150 μL) was added to the cells. After transfer to Eppendorf tubes, the proteins were denatured at 95 °C for 5 min.

Immunodetection of Labeled Proteins. Aliquots of the cellular protein (15 mL) and the standard proteins (20 mL) were run on 10% SDS−PAGE at 200 V until the dye front reached the bottom of the gel (*28*). Proteins were blotted on nitrocellulose at a constant current of 54 mA (1.2 mA/cm^2) using a semidry system. Transfer of protein was checked by staining with Ponceau-S. After thorough washing with TBST (Tris-buffered saline containing 0.2% Tween-20) and TBST/0.2% Protifar, blocking was performed with TBST/2% Protifar. Immunodetection of labeled proteins was performed with an HRP-conjugated polyclonal antibody against fluorescein (1:1000 (v/v) in TBST/0.2% Protifar) for 1 h at room temperature followed by Enhanced Chemiluminescent (Amersham) detection according to instructions. Labeled proteins were visualized by exposing Hyperfilm MP (Amersham) to the blot for 2−5 min.

RESULTS

Labeling of Standard Proteins. The ability of TyrFluo to couple to proteins was established by measuring its cross-linking efficiency to a set of standard proteins under various oxidative conditions. As shown in Figure 2 (panel A) each of the five proteins present were covalently linked to TyrFluo upon exposure to H_2O_2/Fe^{2+} (lane 1), to SIN-1 (lane 2), or HRP/H_2O_2 (lane 3). Under nonoxidative conditions no labeling by TyrFluo of any of the proteins present was

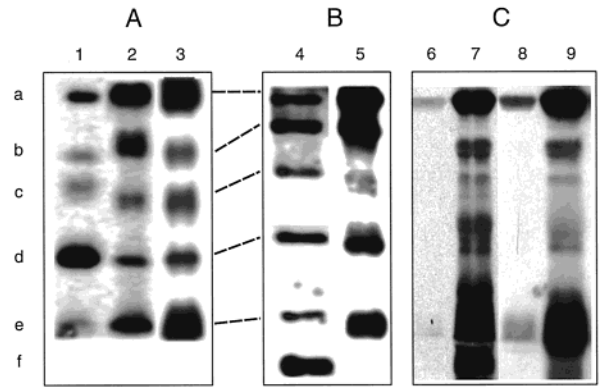

FIGURE 2: ROS-induced labeling of protein standards by TyrFluo. Panel A: proteins a to e after exposure to either 100 μM H_2O_2/100 nM Fe^{2+} (lane 1), 100 μM SIN-1 (lane 2), or to 16 nU HRP/10 μM H_2O_2 (lane 3). Panel B: Proteins a to f exposed to 100 mM CumOOH/100 nM hemin. Prior to detection of TyrFluo-labeled proteins with peroxidase-coupled anti-fluorescein antibody (lane 5) proteins were stained with Ponceau-S (lane 4). Panel C: Proteins a to f after exposure to 16 nU HRP/10 μM H_2O_2 (lanes 6−7) or to 100 mM SIN-1 (lanes 8−9) in the presence (lanes 6, 8) or absence of 500 μM tyramine. (a) phosporylase B, (b) bovine serum albumin, (c) ovalbumin, (d) carbonic anhydrase, (e) trypsin inhibitor, and (f) pre-nsL-TP.

observed (data not shown). In another experiment, a protein lacking tyrosine (pre-nsL-TP) (*27*) was added to the standard proteins and in the presence of TyrFluo exposed to CumOOH/hemin. As shown in panel B, all proteins, except pre-nsL-TP, were labeled suggesting that labeling involved the formation of a dityrosine bond between the proteins and TyrFluo as a result of ROS-induced tyrosyl radical formation. In agreement with this, the labeling efficiency was negligible when the experiments were carried out in the presence of an excess of free tyramine (100 fold compared to TyrFluo; panel C, lanes 6 to 9). At certain conditions of oxidation labeling of pre-nsL-TP was observed (panel C, lane 7). This implies that in this case TyrFluo has formed a bond with an amino acid different from tyrosine. It is known that hydroxyl radicals may convert phenylalanine into *o*-tyrosine and *m*-tyrosine (*7*), which may then react with oxidized TyrFluo.

The labeling efficiency differs for the various proteins and varies with the oxidant used (see Figure 2). This has been further investigated by exposing these proteins to increasing concentrations of the oxidants (Figure 3). Under most conditions phosphorylase B was most extensively labeled in line with this protein having the highest number of tyrosine residues (i.e., a total of 36). For comparison, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor have 18, 10, 7, and 4 tyrosine residues, respectively. In general, labeling of these proteins was lower. However, when H_2O_2 was present, carbonic anhydrase was a preferred target (panel A and C) (see Discussion). Labeling of the proteins increased with increasing ROS concentration. In some instances, labeling reached a plateau (panel B) suggesting that the number of tyrosine residues modified was maximal. In other instances, labeling decreases at high ROS concentrations (panel A and D). This may be due to dityrosine formation between probe molecules, thereby making TyrFluo unavailable for reaction with the proteins, and/or to oxidative damage of the fluorescein moiety thereby interfering with the immunodetection.

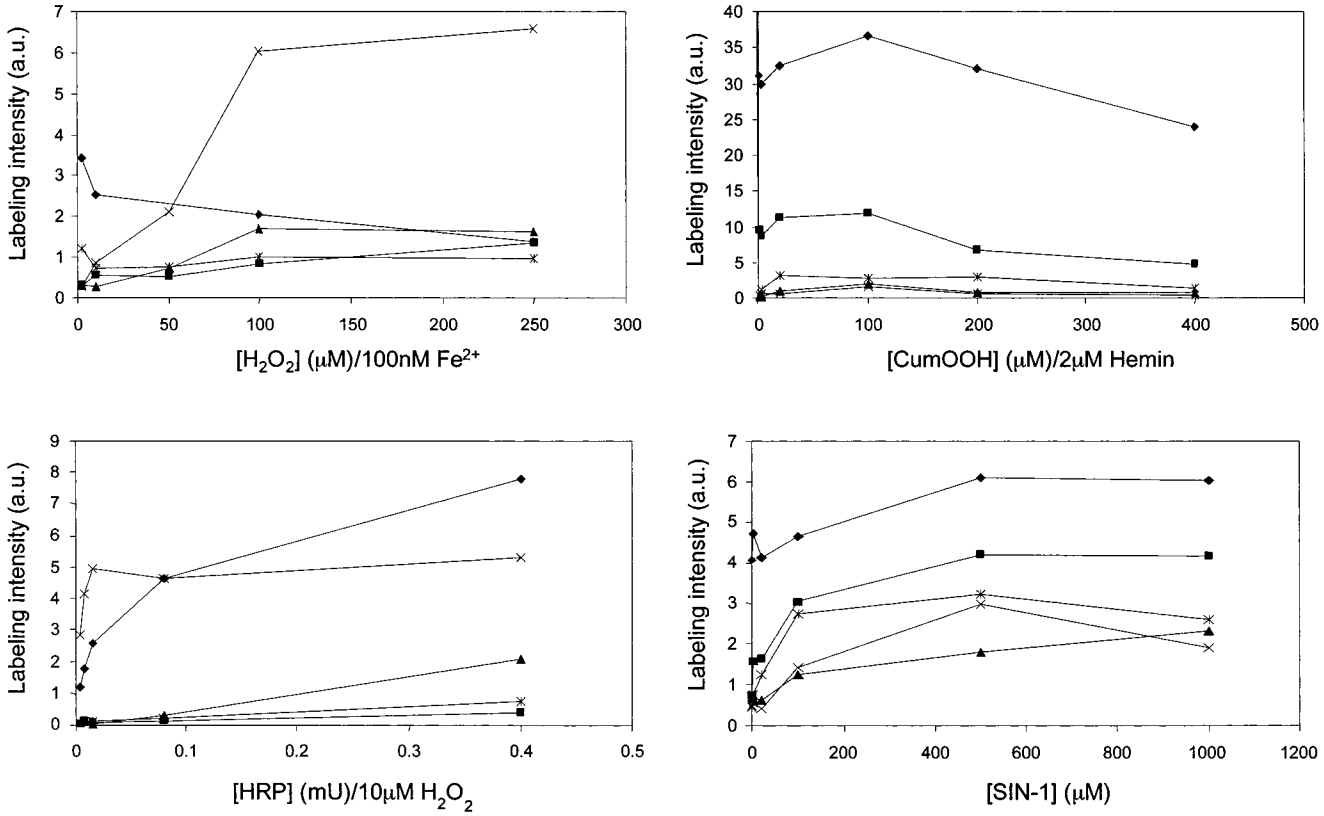

FIGURE 3: Labeling of proteins as a function of ROS concentration. In the presence of TyrFluo (5 mM), the proteins (6 mg each) were exposed to increasing concentrations of H_2O_2/100 nM Fe^{2+} (panel A), CumOOH/2 mM hemin (panel B), HRP/10 μM H_2O_2 (panel C), or SIN-1 (panel D) for 1 h at 37 °C. The intensity indicated was determined by densitometer. -◆-, phosphorylase B; -▲-, ovalbumine; -■-, bovine serum albumin; -×-, carbonic anhydrase; -★-, trypsin inhibitor.

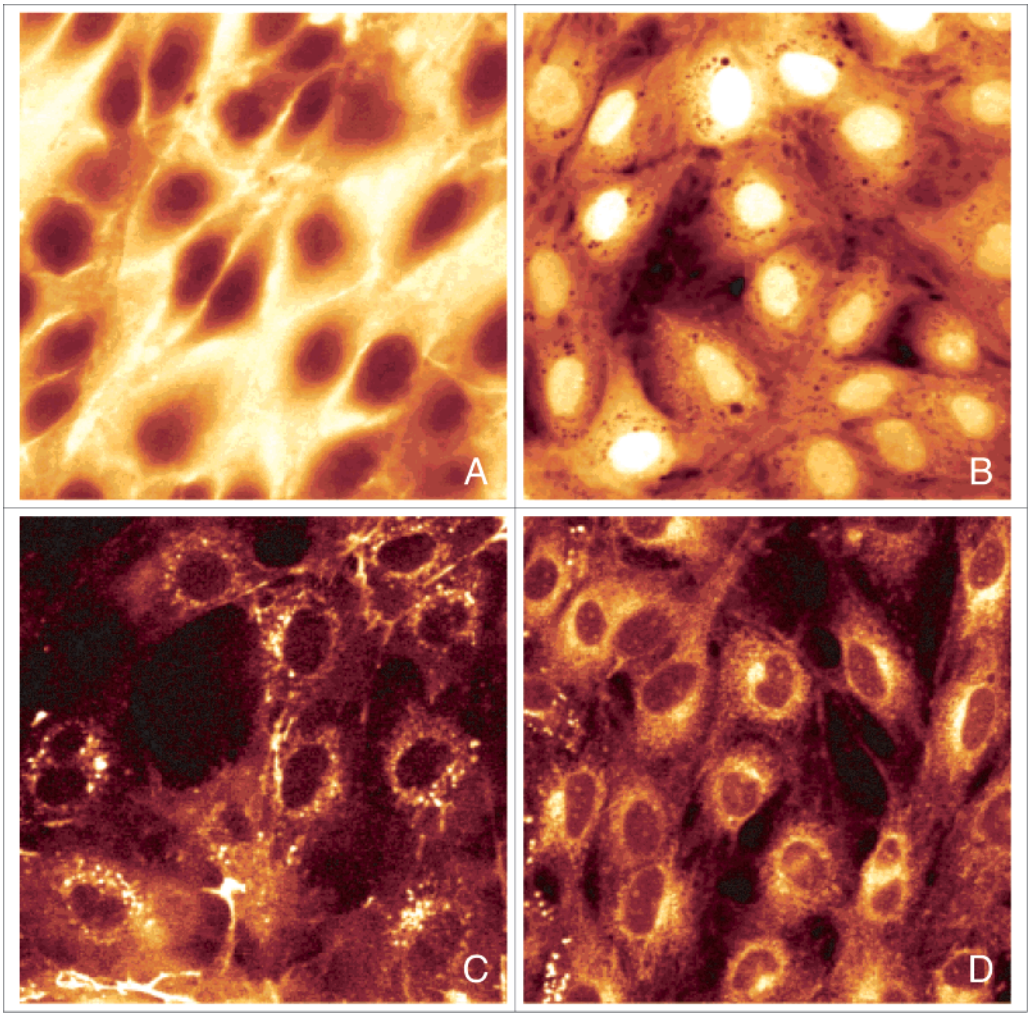

FIGURE 4: Fluorescent micrographs of rat-1 fibroblasts before and after exposure to oxidative stress in the presence of TyrFluo or acetylTyrFluo. Cells were incubated with TyrFluo (panel A) and then stressed with 0.2 mU HRP/1 μM H_2O_2 (panel C) or the cells were preincubated with acetylTyrFluo (panel B) and then challenged with 100 μM CumOOH/100 nM hemin (panel D). Incubations were for 15 min at 37 °C. Labeling of proteins was visualized after removal of noncovalently bound probe (panels C and D). The cells were washed with PBS. Proteins were fixed with 4% paraformaldehyde/0.1% Triton X-100 in PBS.

Labeling of Proteins in rat-1 Fibroblasts. Addition of the probes to cells shows that TyrFluo was outside the cell (Figure 4, panel A), whereas acetylTyrFluo was membrane permeable accumulating inside the cell (panel B). In the latter case, the probe may be deacetylated preventing it from leaving the cell. This different localization has been further explored by exposing cells to HRP/H_2O_2 in the presence of TyrFluo so as to generate tyrosyl radicals extracellularly. After fixation of the cells a staining pattern was observed which may represent proteins labeled at the plasma membrane (panel C). The punctuated pattern may indicate that labeled protein has been internalized by endocytosis. When cells were preincubated with acetylTyrFluo and then exposed to CumOOH/hemin so as to generate tyrosyl radicals inside the cell (panel D) a completely different labeling was observed representing the tyrosylation of intracellular proteins (panel D). Stressing the cells by addition of H_2O_2 gave a staining comparable to that of CumOOH/hemin (results not shown). Surprisingly, despite the localization of the acetylTyrFluo in the nucleus (panel B) the labeling of nuclear proteins was very little (panel D). This suggests that the nucleus may be well protected against radical production including tyrosyl radicals.

Identical experiments were carried out to obtain labeled proteins to be analyzed by Western blotting using an antibody against the fluorescein moiety. As shown in Figure 5, stressing the fibroblasts preloaded with acetylTyrFluo, with both H_2O_2 (lane 2) and CumOOH/hemin (lane 3) gave rise to the labeling of a distinct set of proteins. As one would expect these proteins are different from the proteins labeled from the outside when the cells are exposed to HRP/H_2O_2 in the presence of TyrFluo (lane 5). Under both labeling conditions, the tyrosylated proteins do not coincide with the overall protein staining (lanes 1 and 4). Some proteins that were heavily labeled (see arrows in Figure 5) were barely detectable by protein staining.

DISCUSSION

To date, the detection of dityrosine formation as a measure of protein oxidation has been carried out by HPLC (*6*, *29*), mass spectrometry (*7*), and time-resolved fluorescence studies (*30*). Here we present a novel, sensitive method for the detection of ROS-induced protein tyrosylation in cells. In this method, use is made of a probe consisting of a tyramine moiety covalently coupled to a fluorescein moiety. Tyramineresembles tyrosine except that the carboxyl moiety is

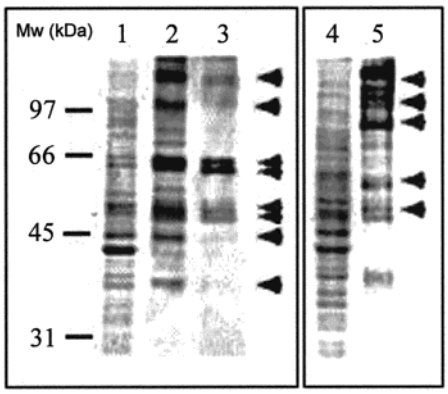

FIGURE 5: Exposure of rat-1 fibroblasts to different oxidants in the presence of acetylTyrFluo or TyrFluo leads to a characteristic pattern of protein labeling. In the presence of acetylTyrFluo, cells were exposed to 100 μM H_2O_2 (lane 2) or to 100 μM CumOOH/100 nM hemin (lane 3). In the presence of TyrFluo cells were exposed to 0.2 mU HRP/1 μM H_2O_2 (lane 5). Overall protein staining by Ponceau-S (lanes 1 and 4). The arrows indicate the major proteins labeled.

lacking. In the presence of ROS, both tyramine and tyrosine as part of proteins may become oxidized enabling the tyrosyl radicals generated to form an *o,o′*-dityrosine bond between probe and protein (here designated as protein tyrosylation). The presence of fluorescein makes that the tyrosylated proteins can be visualized in cells by fluorescence microscopy and on a blot by immunodetection using an anti-fluorescein antibody. In the present study, we have used TyrFluo to label cell surface proteins. Acetylation of TyrFluo makes the probe membrane permeable so as to label intracellular proteins. Upon uptake into the cell, acetylTyrFluo becomes deacetylated by esterases ensuring that the probe is present in the cell during ROS-induced oxidation (*31*).

To determine the sensitivity for tyrosylation a set of standard proteins (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase II, trypsin inhibitor) was exposed to various oxidative conditions (H_2O_2/Fe^{2+}, SIN-1, HRP/H_2O_2, and CumOOH/hemin) in the presence of TyrFluo. Under each of these conditions, the proteins became labeled indicating that both tyramine and tyrosine residues were converted into tyrosyl radicals and available to form an *o,o′*-dityrosine bond. Tyrosyl radical formation implies that each of the oxidative conditions used is suited to abstract a hydrogen atom from the aromatic ring. When these reactions were carried out in the presence of an excess of free tyramine (100 fold compared to TyrFluo) the labeling efficiency was greatly reduced showing an effective competition between free tyramine and TyrFluo for the tyrosyl radicals on the protein.

Under most conditions phosphorylase B was most extensively labeled in line with this protein having the highest number of tyrosine residues. In contrast, ovalbumin, a multimeric protein containing 40 tyrosine residues (divided over four identical subunits) was much less labeled (Figure 2). A determining factor will be the susceptibility of tyrosine residues to become oxidized that may differ for each protein. The number of tyrosine residues that are solvent exposed can be estimated for each protein from its 3D-structure using the Swiss PDB Viewer. For example, if the fraction of the tyrosine surface exposed is set at 15%, phosphorylase B exposes 14 tyrosine residue as compared to 6 for bovine serum albumin, 3 for carbonic anhydrase, 1 for trypsin inhibitor, and 1 for ovalbumin subunit. At this limit (set arbitrarily), the extent of labeling agrees reasonably well with the number of tyrosine residues (cf. Figure 3). Although the exposure to the solvent must be a factor, in the end it is the ability of the TyrFluo radical to interact with the tyrosyl radical on the protein that determines whether labeling occurs. Moreover, the oxidation condition itself may have an effect on the extent of labeling as seen with carbonic anhydrase treated with H_2O_2 (see Figure 3). A special feature of carbonic anhydrase is its zinc-binding domain. This domain may be involved in the formation of hydroxyl radicals from H_2O_2 by the Fenton reaction, thereby enhancing the extent of labeling (*32*). It remains to be established whether the labeling by TyrFluo is restricted to *o,o′*-dityrosine bond formation. Multiple tyrosylations on one tyrosine residue have been reported yielding trityrosine, isodityrosine, or pulcherosine (*33*), which may play a role in the observed difference in labeling efficiency of the proteins. Moreover, hydroxyl radicals may convert phenylalanine into *o*-tyrosine and *m*-tyrosine. This may explain that under certain conditions pre-nsL-TP, void of tyrosine residues, becomes labeled.

Exposure of rat-1 fibroblasts to oxidative stress in the presence of either membrane-impermeable TyrFluo or membrane-permeable acetylTyrFluo gave a cellular labeling characteristic for each probe (Figure 4, panels C and D). Provided labeling by TyrFluo is restricted to plasma membrane proteins exposed to the medium, one may assume that in time these labeled proteins are taken up by endocytosis (*34*). This may explain the punctuated labeling pattern representing labeled endosomes (panel C). Western blot analysis confirmed that upon oxidative stress each probe labeled a characteristic set of proteins (Figure 5), and that also here the presence of an oxidant is a prerequisite for protein labeling. In agreement with what we have observed with the standard proteins, the labeling efficiency of each cellular protein was different most likely reflecting the susceptibility to oxidation by ROS. In addition, the tyrosyl radical formed must be accessible to the oxidized probe. The fact that H_2O_2 added to cells is sufficient to cause a labeling of proteins may be explained by the presence of intracellular transition metals converting H_2O_2 into hydroxyl radicals (*16*). Another possibility is that H_2O_2 may serve as a substrate for intracellular peroxidases and, when oxidized, readily utilizes tyrosine as a reducing substrate and yield a tyrosyl radical. ROS-induced labeling of proteins by TyrFluo may mimic the tendency of oxidized proteins to form intermolecular dityrosine bonds (*13*, *14*). We have to await the identification of the labeled proteins to establish which proteins are particularly sensitive to ROS-induced oxidation. This identification may give further insight in the effects of their oxidation and subsequent loss of function as it relates to cellular disorders and aging.

ACKNOWLEDGMENT

We thank Ginette Ploeger and Elke Pröbst-Biegelman for their contributions to this study.

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BI002795S