Dual oxidase 2 is expressed all along the digestive tract.

Rabii Ameazine El Hassan, Rabii, Nesrine Benfares, Bernard Caillou, Monique Talbot, Jean-Christophe Sabourin, Virginie Belotte, Stanislav Morand, Sedami Gnidehou, Diane Agnandji, Renee Ohayon, Jacques Kaniewski, Marie-Sophie Noel-Hudson, Jean-Michel Bidart, Martin Schumberger, Alain Virion, and Corinne Dupuy

1Unité 486 INSERM, Université Paris 11, Faculté de Pharmacie, Châteenay-Malabry Cedex and 2UMR 8125 CNRS/CEA LRC 29V, Institut Gustave-Roussy, Villejuif, France

Submitted 29 April 2004; accepted in final form 23 November 2004

Dual oxidase 2 is expressed all along the digestive tract. Am J Physiol Gastrointest Liver Physiol 288: G933–G942, 2005. First published December 9, 2004; doi:10.1152/ajpgi.00198.2004.—The oxidase gene; NADPH oxidase; hydrogen peroxide

thyroid gland; gastrointestinal system; dual oxidase gene; thyroid oxidase gene; NADPH oxidase; hydrogen peroxide

REACTIVE OXYGEN SPECIES (ROS) have emerged as important molecules in many cells and are involved in regulating essential cell functions such as growth and differentiation (2). In the thyroid, H2O2 is the final electron acceptor for the thyperoxidasen-catalyzed biosynthesis of thyroid hormone at the apical surface of the thyrocytes (28).

A functional NADPH oxidase, generating H2O2 in a Ca2+-dependent manner, has been solubilized from pig thyroid (13), and a flavoprotein with an apparent molecular mass of ~180 kDa has been purified from it (9). Microsequences were used to clone its porcine and human partial cDNAs (9). The full-length cDNA, encoding a 1,548-amino acid protein known as thyroid oxidase 2 (Thox2), has subsequently been cloned from human thyroid (5). Its sequence is 83% similar to that of the Thox1, which is also expressed in the thyroid gland (5). Thox1 and 2 proteins and THOX1 and 2 genes are also known as dual oxidases 1 and 2 (Duox1 and 2) and DUOX1 and 2 genes.

Duox1 and Duox2 are the long homologs of gp91phox, the catalytic core of phagocytic NADPH oxidase that generates superoxide when phagocytic cells ingest bacteria. They belong to a new family of seven NOX/DUOX genes, which encode seven different NADPH oxidases and have differing mRNA tissue expressions (19). Although a functional Duox-based H2O2-generating system has not yet been reconstituted (6), the essential role of Duox2 in thyroid hormone synthesis has been confirmed by the recent observation of permanent and severe congenital hypothyroidism in a patient with a biallelic inactivating mutation in the DUOX2 gene (24).

DUOX2 gene expression is not restricted to the thyroid, and it has also been found in the rat colon by RT-PCR (10). Recently, Leto et al. (11) reported high DUOX2 mRNA expression in the salivary gland and rectum by Northern blot analyses and visualized DUOX2 expression in rectal epithelial cells by in-situ hybridization experiments. In the same study, small amounts of DUOX2 mRNA were detected by Northern blot analysis in the cecum and ascending colon. We present here data demonstrating that the Duox2 protein is expressed in all segments of the porcine digestive tract, but in a particularly marked degree in the large intestine. This expression was associated with NADPH-dependent H2O2-generating activity that has the same biochemical characteristics as thyroid NADPH oxidase. We also show that the Duox2 protein is expressed in the human colon, small intestine, and duodenum as well as in a human colon adenocarcinoma cell line (Caco-2).

MATERIALS AND METHODS

Cell culture. Cultured human colonic adenocarcinoma Caco-2 cells, kindly donated by Dr I. Beaumont (INSERM U 510, Châteenay-Malabry, France), were grown in DMEM (high glucose) medium (Invitrogen) supplemented with 15% FCS, 1% penicillin, 1% streptomycin, and 1% Fungizone. The cells were maintained at 37°C in a 10% CO2-90% air mixture. The culture medium was changed daily.

Preparation of the particulate fractions. Fresh tissues from two pigs were obtained from Institut National de la Recherche Agronomique and conveyed to the laboratory on ice. Normal human colon tissues were obtained from surgical specimens at Institut

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpgi.org 0193-1857/05 $8.00 Copyright © 2005 the American Physiological Society G933
Gustave Roussy in accordance with local and national ethical laws and conveyed to the laboratory on ice. All procedures were carried out at ~4°C. The porcine digestive tract was divided into eight segments: stomach, duodenum, jejenum, ileum, cecum, sigmoidal colon, floating colon, and rectum (Fig. 1). The tissues were cut into small pieces and suspended in three volumes of 0.25 M sucrose, 50 mM phosphate buffer, pH 7.20, 1 mM EGTA, 2 mM MgCl2, and protease inhibitors (5 μg/ml leupeptin, 0.15 mg/ml benzamidine, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 16 mg/ml PMSF). The suspension was then homogenized with an Ultra-turrax for 2 min. The homogenate was filtered through six layers of cheesecloth and centrifuged at 500 g for 15 min in a Sorvall SS34 rotor. The supernatant obtained was then centrifuged at 3,000 g for 15 min in the same rotor. The pellet was washed twice with three volumes of buffer A (0.25 M sucrose, 50 mM phosphate buffer, pH 7.20, 2 mM MgCl2, and protease inhibitors) and centrifuged at 3,000 g for 15 min at 4°C. The final pellet was gently suspended in 0.5 volumes of buffer A using a glass/Teflon potter homogenizer to provide the particulate fraction.

RNA isolation and Northern blot analysis. RNA was extracted from all the tissues and from the Caco-2 cells by the method of Chomczynski and Sacchi (4). Northern blot analyses were performed as previously described (9). Final washes were carried out at 60°C in 0.1% SSC, 0.1% SDS (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate). The porcine cDNA probe used was DUOX2 cDNA prepared by RT-PCR, using total RNA from pig thyroids. The sense and antisense DUOX2 primers, designed on the basis of the 3′-untranslated region (UTR) of the DUOX2 cDNA, were 5′-CAGTTCAATCTGGACTC-AA-3′ and 5′-GAGCCAAACGATCACTGAGC-A-3′, respectively. The human cDNA probe used was DUOX2 cDNA prepared by RT-PCR using total RNA from human thyroids. The sense and antisense DUOX2 primers, designed on the basis of the 3′-UTR of the DUOX2 cDNA, were 5′-TGGCAAGGCCGTTGGAACCAAA-3′ and 5′-CACATCACTGTTGGTTCT-3′, respectively. The cDNA probes were α-32P-labeled by random priming extension using a kit (Amersham). Membranes were analyzed by electronic autoradiography using INSTANTMAGER (Packard).

RT-PCR experiment. Total RNA (2 μg) was treated with 15 U ThermoScript RT (Invitrogen) in 20 μl PCR buffer for 90 min at 60°C according to the manufacturer’s protocol. The control run was without the RT. The porcine DUOX2 cDNA and DUOX1 cDNA were amplified by 30 temperature cycles (95°C, 5 s; 62°C, 10 s; 72°C, 2 min) in a GeneAmp 2400 temperature cycler (Perkin Elmer) in 50 μl pre-warmed PCR buffer containing 1 μl of Advantage 2 polymerase Mix (Clontech), 250 nM of each sense and antisense oligonucleotide primer, and 200 μl of each dNTP. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA was amplified in parallel. The sense and antisense PCR primers for porcine DUOX2 were designed from the porcine DUOX2 cDNA sequence and were 5′-CAGTTCAATCTGGACTCAAA-3′ and 5′-GAGCCAAACGATCACTGAGCAA-3′, respectively. The amplified product was expected to contain 1051 bp. The sense and antisense PCR primers for porcine DUOX1 cDNA were 5′-TCAAGGCCGGAGCTGTTGGA-3′ and 5′-GGCAGTGGCTCCGTTTGGTC-3′, respectively, and the amplified product was expected to contain 954 bp. The sense and antisense primers for G3PDH were 5′-ACCACGTGCTCACTCAGCA-3′ and 5′-TCCACACCGCTTTGCTGTA-3′, respectively, and the amplified product was expected to contain 452 bp.

Cloning of the human extracellular domain of Duox2 and bacterial expression. The extracellular domain encompassing the G21-L589 fragment of the human Duox2 (ECD) was produced in Escherichia coli by pTrcHis TOPO TA expression kit (Invitrogen). The expression of the nucleotide was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside to the bacterial culture for 4 h. After being centrifuged at 4,000 g for 20 min, the bacterial pellet was resuspended in 20 ml of the lysis buffer (50 mM HEPES-NaOH, pH 7.5, 0.5 mM NaCl, 1 mM PMSF, and 5 mM DTT) containing 0.35 mg/ml lysozyme and sonicated for 10 min on ice. One milligram of DNAse I was added, and the suspension was shaken for 1 h at 37°C. The bacterially expressed protein located in inclusion bodies was obtained by centrifuging at 30,000 g for 30 min at 4°C. The pellet was washed twice with PBS containing 1% Triton X-100 and resuspended in 3 ml 50 mM HEPES-NaOH, pH 7.5, 6 M guanidine/HCl, 1 mM DTT, and 0.2% sarcosyl. Proteins were loaded onto a nickel chelator column (Probind, Invitrogen) preequilibrated in buffer P (20 mM sodium phosphate buffer, pH 7.8, containing 4 M urea, 0.2% sarcosyl, 2 mM 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), and 0.5 M NaCl). The column was washed stepwise using buffer P at pH values of 6, 5.3, and 4. The bound, His-tagged protein was eluted with 50 mM EDTA, pH 7.6, and concentrated 10-fold on centrifcon 50 (Amicon) before loading onto a 4–12% polyacrylamide gel. After electrotransfer the SDS-PAGE gel was transferred into a 0.3 M solution of CuCl2 and shaken for 2 min. The band of interest, visualized on a black background, was cut out, placed in a syringe, and forced through the nozzle without the needle into a second syringe according to the method previously described (26). This was repeated five times. The gel material was collected in a 2-ml Eppendorf tube with 20 mM sodium phosphate buffer, pH 8, containing 4 M urea and 100 mM EDTA. The mixture was vortexed and incubated at room temperature for 15 min and then poured onto a small column (Pierce). The protein was eluted from the gel by adding 20 mM sodium phosphate buffer, pH 8, containing 0.4 M urea and 0.01% sarcosyl and concentrated 10-fold on centrifcon 50 (Amicon).

Western blot analysis. Protein samples (40 μg) were suspended in the sample buffer (2% SDS, 5% β-mercaptoethanol, and 10% glycerol), and SDS-PAGE and immunoblot analyses were performed as previously described (3). Briefly, an anti-Duox antibody raised against a 14- amino acid peptide encompassing the L410-G423 portion of human Duox2, which is 100% conserved in porcine Duox2, was used to probe the immunobots. A previous study (23) investigating the specificity of this antipeptide toward porcine Duox1 and Duox2 proteins by Western blot analysis has shown that it essentially recognizes the pig Duox2 protein. A rabbit polyclonal antibody was raised against the Glu639-Arg1039 fragment of Duox2 produced in E. coli by pTrcHis TOPO TA expression kit (Invitrogen). It was produced by Eurogentec (Seraing, Belgium). This antibody was used to probe the immunoblot of human proteins at a dilution of 1:5,000. It was used at a dilution of 1:200 in immunohistochemistry experiments on human tissues. Monoclonal antibodies raised against the human ECD were prepared as described in the following protocol. BALB/c mice were

Fig. 1. Segmentation of the porcine gut.
injected subcutaneously with 18 μg ECD in 100 μl complete Freund adjuvant, followed by three booster injections with 18, 20, and 50 μg ECD in 100 μl incomplete Freund’s adjuvant (Difco) at 3-, 4-, and 1-mo intervals, respectively. Finally, mice were injected intraperitoneally with 50 μg ECD in 100 μl incomplete Freund’s adjuvant. Ten days later, their spleens were harvested and hybridoma was prepared. The hybridoma supernatants were evaluated by Western blot analysis on membrane extract of the stable human DUOX2-expressing HEK-293 cell line established using the Flp-in system (Invitrogen). Isotyping test revealed that MAbs were IgG2b isotypes. The monoclonal antibody was used to probe the immunoblot at a dilution of 1:500.

Immunohistochemistry. Immunohistochemistry was performed on Duboscq-Brasil-fixed, paraffin-embedded tissue blocks of tissue samples as previously described (3). Normal human tissues were fixed in acetic acid, formaldehyde, and alcohol. Negative controls were prepared by incubating tissues with preimmune antisera.

Construction of the human ECD of Duox2 deletions. Deletions of various regions of the ECD were created using the “QuickChange” site-directed mutagenesis kit from Stratagen, with the ECD-pTrcHis-TOPO vector as a template. The 1–122 deletion was created using 5′-GATGACGATAGATCCACACTTGGTGTCGAGTTCCAAGCTTATAGCTCATC-3′ and 5′-GGTTGGAATCTCGGCGGACACCATGGGTTGCCCCGCCGAG-3′ as mutagenic oligonucleotides. The oligonucleotides used for the 123–224 deletion were 5′-GACGTTGGATGCTGCTGGCCACATGGGGCTTCTACCGCTACGTG-3′ and 5′-GTCTGTCGCGTTCGCGGCCCAGC-3′ and 5′-GGGGCTTCTACCGCTACGTG-3′ as mutagenic oligonucleotides used for the 225–326 deletion were 5′-ACACCCTGTGCTATGTTGCGCGGAAATTTGTTGCGCTTGACAG-3′ and 5′-CTGCTCAGGAGGCACACAAATTCGGCGGCCCACATAGCGAGGTTG-3′. The oligonucleotides used for the 327–424 deletion were 5′-TTCCTAGACCCCCACATCCTCCTGATTGCGGAGCATTACACGTG-3′ and 5′-ACGTTGGATGCGTGTGCGCCACATACGCGGAGATGCTGGGTCACGGA-3′. All deletions were confirmed by sequencing.

Stable cell transfection. The stable human DUOX1- and DUOX2-expressing cell lines were established using the FLP-in system (Invitrogen). The protocol accompanying the kit was used without modification. Human DUOX1 and DUOX2 cDNAs were subcloned in pcDNAs/FRT vector (Invitrogen) designed for use with the FLP-In System. When cotransfected with the pOG44 Flp recombinase expression plasmid into a Flp-In-293 cell line, the pcDNAs/FRT vector containing the DUOX1 or DUOX2 cDNA was integrated in a Flp recombinase-dependent manner into the genome. Stable cell lines were established by exposure to 100 μg/ml hygromycin.

Preparation of the plasma membrane-enriched fraction from pig thyroid gland. The preparation of plasma membranes was prepared as described previously (21).

Determination of protein content. Protein concentration was determined by the Bradford method (1).

Measurement of NADPH oxidase activity. Particles were incubated at 30°C in 1 ml 200 mM sodium phosphate buffer, pH 7.4, containing 1 mM sodium azide, 0.5 mM CaCl₂, 0.4 mM EGTA, 0.1 μM FAD, and 0.1 mM NADPH. Eight aliquots (100 μl) of each sample were collected at time intervals between 0 and 20 min and mixed with 10 μl 3 N HCl to stop the reaction and destroy the remaining NADPH. Fluorescence (excitation: 360 nm, emission: 460 nm) was measured in a Perkin Elmer MPF 43A spectrofluorimeter after adding to each aliquot 2 ml 200 mM sodium phosphate buffer, pH 7.8, containing 0.25 μM scopein and 5 μg/ml horseradish peroxidase. The concentration of H₂O₂ was directly correlated to the concentration of oxidized (nonfluorescent) scopein.

Treatment of the particulate fraction with phenylarsine oxide. The particulate fractions were treated as previously described (14). Briefly, the particulate fraction (1 ml, 100–120 μg/ml) was preincubated for 10 min at 0°C in 50 mM sodium phosphate, pH 7.25, containing 0.5 mM CaCl₂, 0.4 mM EGTA, 1 mM NaN₃, 10 μM FAD, and 10 μM CHAPS. The final pellet was resuspended in 50 μl of 200 mM sodium phosphate buffer, pH 7.25, containing 0.5 mM CaCl₂, 0.4 mM EGTA, 1 mM NaN₃, and 10 mM CHAPS, and the residual activity was measured immediately. The final pellet was resuspended as before but without Ca²⁺ and with 7.5 mM EGTA to study the effect of the absence of Ca²⁺.

Solubilization. Particulate fractions of porcine sigmoidal colon were thawed on ice and added with 1% Triton X-100 or 2 M KCl or both, respectively. The suspensions were gently stirred for 60 min at 4°C and centrifuged at 200,000 g for 30 min. The supernatant was removed, frozen quickly in liquid nitrogen, and stored at −80°C until used.

RESULTS

Detection of porcine DUOX2 along the digestive tract by Western blot analysis. Immunoblot analysis of membrane proteins from different tissues of the digestive tract shows that the protein Duox2 was expressed in all the tissues tested, but to a much greater extent in the stomach, cecum, and sigmoidal colon (Fig. 2).

Western blot analysis showed that membrane proteins displayed Duox2 as two bands at 165 and 175 kDa, respectively, as previously reported for the pig thyroid (23). These two proteins correspond to two differently N-glycosylated forms of Duox2 (6). It was shown that only the more highly glycosylated form was resistant to endoglycosidase H digestion, indicating its passage through the Golgi apparatus and also suggesting that it constitutes the mature form involved in the active NADPH oxidase at the plasma membrane (6). As observed in the thyroid, the 165-kDa form, which is the precursor of the 175-kDa form, was more abundant. Preincubating the antibody with an excess of synthetic peptide prevented the labeling of these proteins (data not shown).

Expression of Duox2 protein in digestive tissues. Figure 3 shows an immunohistochemical comparison of Duox2 protein expression in porcine thyroid and cecum. As previously shown in human thyroid (3), Duox2 protein was detected at the apical membrane of thyrocytes (Fig. 3A). Unlike human thyroid, the porcine tissue was homogenously stained and positive cells were contiguous, indicating that the mature form of Duox2 was expressed in all the tissues tested.
expressed to a greater extent in porcine tissue. This finding was in accordance with previous findings showing that the 175-kDa form is specifically detected in a porcine thyroid fraction containing 10–20 times more NADPH oxidase activity than the human particulate fraction (21). In the porcine digestive tract, immunohistochemistry revealed intense staining at the apical surface of the epithelial cells of the cecum, with greater expression at the surface epithelium (Fig. 3, B and C). No staining was seen at the bottom of the crypt. These findings indicated that Duox2 protein was only expressed in highly differentiated cells.

The comparison of immunostaining for Duox2 on serial tissue sections from different gastrointestinal tissues is shown in Fig. 4. In these tissues, staining was observed at the brush border of the enterocytes, confirming that expression of Duox2 was restricted to highly differentiated enterocytes. At high magnification, immunostaining was also detected in the perinuclear zone, probably corresponding to the presence of Duox2 in the Golgi apparatus during maturation (Fig. 4B).

Analysis of DUOX2 mRNA expression along the digestive tract. We studied DUOX2 mRNA expression in the same tissues by Northern blot analysis and compared its expression to that of the protein. Northern blot analysis detected higher levels of DUOX2 mRNA in the porcine cecum than in the thyroid. The DUOX2 transcript was also abundant in the sigmoidal colon (Fig. 5A). These results matched the protein-expression profile. On the other hand, DUOX2 mRNA was not detected by Northern blot analysis in the other tissues, even those in which the Duox2 protein appeared to be well expressed, such as in the stomach and floating colon. DUOX2 mRNA was, however, detectable by RT-PCR (Fig. 5B), and it was concluded that DUOX2 transcript levels must vary considerably in different tissues and not necessarily be related to protein expression. We also compared DUOX1 and DUOX2 mRNA expression in gastrointestinal and thyroid tissues. For this purpose, PCR experiments were performed to generate amplification products of similar size with oligonucleotides in the specific 3′-UTR of DUOX1 and DUOX2 mRNAs. Figure 5B shows that DUOX1 mRNA was not at all, or only very weakly, expressed in gastrointestinal tissues, whereas it was clearly detected in the thyroid gland.

Expression of Duox2 protein in human tissues. Figure 6A shows an immunohistochemical comparison of Duox2 protein expression in human colon, small intestine, and duodenum. Immunohistochemistry showed that staining was more pronounced on the surface of epithelium. As observed with the porcine colon, the labeling was much more pronounced on the apical membrane. Western blot analysis of surgically removed specimens from four patients showed that membrane proteins displayed Duox2 as a band at 165 kDa (Fig. 6B). A 165-kDa Duox1/2 protein had already been observed in the 100,000 g pellet from normal human thyroid and after transient transfection of nonthyroid cells (3). The 175-kDa form was not detectable in human colon tissue, as had previously been reported in the human thyroid tissue (3).

Measurement of NADPH-dependent formation of H2O2 in porcine gastrointestinal tissues. The expression of a 175-kDa form of Duox2 suggested the presence of a functional Thox in the gastrointestinal porcine tissues. To confirm this hypothesis, we measured the ability of the particulate fraction from these tissues to generate H2O2 in the presence of NADPH. Thyroid NADPH oxidase requires micromolar concentrations of calcium to acquire a functional conformation and to generate H2O2 (7, 21, 25). Duox2 contains two Ca2+-binding motifs, which could be involved in the direct activation of the H2O2 generator by calcium. Consequently, the NADPH-dependent H2O2-forming activities of gastrointestinal particulate fractions were measured in the presence and in the absence of calcium. As shown in Fig. 7, particles from all the tissues incubated with NADPH generated H2O2; this was Ca2+ dependent as in the thyroid particulate fraction (7). The H2O2-generating activity was correlated to Duox2-protein expression and was much higher in cecum and helicoidal colon tissues, where the highest levels of Duox2 were also found by Western blot
analysis (Fig. 2). The H\textsubscript{2}O\textsubscript{2}-generating activity observed in stomach was only partly dependent on calcium. This could be due to the partial proteolysis of the H\textsubscript{2}O\textsubscript{2}-generating system during the preparation of the particulate fraction from this tissue, which contains particularly high levels of proteases. Indeed, we had previously observed that the NADPH oxidase from the thyroid is fully desensitized to Ca\textsuperscript{2+} after limited proteolysis by \(\alpha\)-chymotrypsin (8).

**Solubilization of the NADPH oxidase.** To determine the subcellular distribution of the H\textsubscript{2}O\textsubscript{2}-generating system, the porcine colon particulate fractions were treated with 2 M KCl with the nonionic detergent Triton X-100 or with both. Figure 8A shows that NADPH-dependent, H\textsubscript{2}O\textsubscript{2}-generating activity was solubilized by a high concentration of salt in the presence of detergent, as previously demonstrated for pig thyroid plasma membrane (13). The enzyme was not extracted from the particulate fractions by either Triton X-100 or KCl alone. As previously observed, the enzymatic activity could only be measured after overnight dialysis (13). In a previous study, we showed that it took at least 6 h to restore the NADPH-dependent, H\textsubscript{2}O\textsubscript{2}-generating activity, suggesting that either a conformational change of the protein or a reassociation between different components extracted by the solubilization procedure had occurred (13). Western blot analysis showed that the Duox2 protein was solubilized under the same conditions (Fig. 8B), indicating that the NADPH-dependent, H\textsubscript{2}O\textsubscript{2}-generating activity measured under these conditions could be ascribed to the Duox2.

**Effect of PAO on H\textsubscript{2}O\textsubscript{2} formation.** Trivalent arsenical PAO reacts to form stable dithioarsine rings with two thiol groups that are either adjacent to each other in the protein sequence or close together in the folded protein (i.e., vicinal dithiols). In a previous study of thyroidal NADPH oxidase (14), it was shown that PAO simultaneously caused partial inactivation of the Ca\textsuperscript{2+}-stimulated enzyme and partial activation of the basal activity, resulting in the complete desensitization of the enzyme activity to Ca\textsuperscript{2+}. These findings suggest that thiol groups are involved in the control of thyroid NADPH oxidase by Ca\textsuperscript{2+} and also provide evidence that thyroidal NADPH oxidase differs from cytochrome b-558, the well-characterized neutrophilic NADPH oxidase, which responded differently to PAO treatment (20). To confirm that the NADPH/Ca\textsuperscript{2+}-dependent H\textsubscript{2}O\textsubscript{2} generator identified in all segments of the digestive tract was the same as thyroidal
NADPH oxidase, we studied the effects of PAO on the H₂O₂-generating activity of the particulate fraction of cecum (Fig. 9A). The initial rate of H₂O₂ formation catalyzed by PAO-treated membranes, measured in the absence of Ca²⁺/H₁₁₀₀₁, increased with the concentration of PAO until a plateau was reached at 12/H₉₂₆₂ M PAO, whereas a symmetrical curve was obtained for the PAO-induced partial inhibition of the Ca²⁺/H₁₁₀₀₁-activated enzyme. Consequently, at PAO concentrations higher than 10⁻¹²/H₉₂₆₂ M, the partially inhibited NADPH oxidase became fully Ca²⁺/H₁₁₀₀₁ independent (Fig. 9B).

Once again, this desensitization to Ca²⁺/H₁₁₀₀₁ of the PAO-treated NADPH oxidase was the same as that observed with thyroidal NADPH oxidase (14).

Expression of Duox2 in a human colon adenocarcinoma cell line (Caco-2). Caco-2 cells spontaneously undergo differentiation in postconfluent cultures. Due to their differentiation potential, these cells constitute a widely used model of entero-cytic differentiation and function. The DUOX2 mRNA expression of postconfluent Caco-2 was analyzed, and marked induction was seen at day 7 (Fig. 10A). The expression of DUOX2 mRNA was not detected in exponentially growing nonconfluent cultures. To investigate Duox2 protein expression in Caco-2 cells, we used monoclonal antibodies prepared against the human extracellular domain of Duox2. The deletion analysis showed that the monoclonal antibodies recognized the region between amino acids 123 and 224 (Fig. 10B). Cloning of human DUOX1 and DUOX2 full-length cDNAs permitted us to stably express each protein in HEK293 cells and to evaluate the specificity of this antibody toward human Duox proteins using Western blot analysis (Fig. 10C). The monoclonal antibody, which did not detect porcine Duox2 (lane 3), cross-reacted with both human Duox proteins (lanes 1 and 2) but essentially recognized the human Duox2 (lane 2). Immuno-blot analysis of membrane proteins from Caco-2 cells showed that the Duox2 protein was detected at day 10 (Fig. 10D). This expression was correlated with an increase in a Ca²⁺-dependent, H₂O₂-generating activity measured at day 10 (Fig. 10E).
DISCUSSION

DUOX genes were identified in the thyroid gland and were found to be essentially expressed in this tissue (5, 9). However, expression of the DUOX2 gene is not restricted to the thyroid cell, and DUOX2 mRNA was also found in the rat colon by RT-PCR (10). Recently, Geiszt et al. (11) have published data concerning the expression of the DUOX2 transcript in the salivary glands and rectum detected by Northern blot analysis and in situ hybridization experiments. They found only very low levels of DUOX2 mRNA in the other gastrointestinal tissues. The results of the present study provide new data concerning the expression of the DUOX2 gene in the digestive tract in terms of protein levels. By using an antibody directed against the NH2-terminal part of the protein, we showed by Western blot experiments that Duox2 is not only expressed in the rectum, but also throughout the digestive tract, with much greater expression in the cecum and colon. Immunohistochemistry experiments showed that the Duox2 proteins are localized at the brush border of the enterocytes, indicating that Duox2 is expressed in highly differentiated cells. The same results were obtained with human colon. Furthermore, an NADPH-dependent H2O2-generating activity was found to be linked to Duox2 protein expression in porcine tissues. By using an antibody directed against the NH2-terminal part of the protein, we showed by Western blot experiments that Duox2 is not only expressed in the rectum, but also throughout the digestive tract, with much greater expression in the cecum and colon. Immunohistochemistry experiments showed that the Duox2 proteins are localized at the brush border of the enterocytes, indicating that Duox2 is expressed in highly differentiated cells. The same results were obtained with human colon. Furthermore, an NADPH-dependent H2O2-generating activity was found to be linked to Duox2 protein expression in porcine tissues. The biochemical characteristics of this H2O2 generator are the same as those of thyroidal NADPH oxidase: it was solubilized under the same conditions, and its calcium-dependent activity was similarly modified by PAO treatment, which induced complete calcium desensitization of the enzyme. These new data constitute further evidence suggesting that Duox2 is implicated in the production of H2O2 catalyzed by calcium-dependent NADPH oxidases. No H2O2 production was measurable in the human colon, unlike in porcine tissues. Because H2O2 production activity may be associated with digestive function, this difference could be due to the physiological state of the human tissues, which were taken from patients who had been fasting for 24 h, whereas the porcine tissues were obtained from normally fed animals. However, a species-related difference in Duox2 expression level and/or enzyme intrinsic activity cannot be excluded. Indeed, we previously observed that the NADPH oxidase activity of particulate fractions from human thyroid was 10–20 times lower than that of equivalent fractions from pig thyroid gland (21).

A discrepancy between the Northern and Western blot analyses findings was observed. DUOX2 transcript levels were not related to protein expression. The Duox2 protein appeared to be well expressed in the digestive tract, but its mRNA level varied in different tissues, and in some of them, it could only be detected by RT-PCR. As the Northern blot experiments were carried out using total RNA and not with polyA+ mRNAs on the one hand, and as long mRNAs transfer less efficiently than shorter ones on the other hand, DUOX2 mRNA was probably at the limit of detection under the experimental conditions used. Moreover, unlike what we had observed with porcine tissues, Northern blot analysis of DUOX2 expression in human gastrointestinal tissues showed low levels of DUOX2 in the cecum and ascending colon and high

Fig. 7. Measurement of the NADPH-dependent H2O2-generating activities in gastrointestinal tissues. Particulate proteins from stomach (lane 1), duodenum (lane 2), ileum (lane 3), cecum (lane 4), sigmoidal colon (lane 5), floating colon (lane 6) and rectum (lane 7) were incubated under conditions described in MATERIALS AND METHODS, with 0.1 mM NADPH and 0.5 mM CaCl2 or EGTA in the presence (filled bars) or absence (open bars) of 0.5 mM CaCl2. Similar data were obtained from 2 independent experiments. The results are expressed as means ± SE of 3 determinations.

Fig. 8. Solubilization of the NADPH oxidase. A: distribution of the NADPH-dependent H2O2-generating activity after solubilization. Porcine colon particulate fractions (F; 300 μl) were treated 1 h with the corresponding buffer and then separated into the pellet (P) and supernatant (S) as described in MATERIALS AND METHODS. Aliquots of all fractions (75 μl) were incubated under conditions described in MATERIALS AND METHODS, with 0.1 mM NADPH and 0.4 mM EGTA in the presence (filled bars) or absence (open bars) of 0.5 mM CaCl2. Similar data were obtained from 2 independent experiments. The results are expressed as means ± SE of 3 determinations. B: Western blot analysis of Duox solubilization. Aliquots of all fractions (50 μl) were treated 1 h with the corresponding buffer and then separated into the pellet (P) and supernatant (S) as described in MATERIALS AND METHODS. Aliquots of all fractions (20 μl) were analyzed by Western blot analysis as described in MATERIALS AND METHODS using the antibody raised against the Glu639-Arg1039 fragment of Duox2.
expression in rectum (11). This difference could be ascribed to the physiological state or fed state of the two species when the tissues were taken.

So far, nontyroid cell lines transfected with DUOX2 cDNAs alone have failed to generate H$_2$O$_2$ (6). The lack of H$_2$O$_2$ production could be related to the absence of the most glycosylated form of the Duox2 protein and/or to the absence of Duox expression at the plasma membrane. Duox expression at the plasma membrane was only obtained in the thyroid cell lines, suggesting that an additional thyroid-specific component is required to reconstitute a functional system. In contrast, Western blot analyses made with proteins from different digestive tract tissues revealed two proteins (165 and 175 kDa), corresponding to two different N-glycosylation states of the Duox2 protein previously detected in the thyroid. The component(s) required for the complete Duox2 maturation must therefore also be expressed in the digestive tract.

The existence of two distinct DUOX genes raises the question of their respective roles. The expressions of their respective mRNA are correlated in vivo in both normal thyroid (3) and in thyroid carcinomas (18) and are controlled in similar ways by thyroid-stimulating hormone in vitro (5). It has been suggested that they could be implicated in a heteropolymERIC structure, with each subunit playing a distinct role in the catalytic reaction. Our data showing that Duox1 is not expressed at all, or only weakly expressed, in the digestive tract consequently demonstrate that Duox2 is functionally independent of Duox1. In contrast, the DUOX1 gene appears to be preferentially expressed along the mucosal surface of the trachea and the bronchi.

Our findings in terms of proteins demonstrate that Duox2 is expressed in highly differentiated colon epithelial cells and is therefore probably implicated in some function of these cells. Duox2 was found to be coexpressed with lactoperoxidase (LPO) in the salivary gland and rectum. It has been proposed that Duox2 could be the source of H$_2$O$_2$ for LPO-catalyzed reactions and, therefore, could be implicated in a host-defense mechanism (11). The presence of Duox2 throughout the digestive tract, and particularly in the large intestine where the microbial flora is most abundant, strengthens this hypothesis.

Interestingly, our data show for the first time that DUOX2 expression is induced during the differentiation of the Caco-2 cell line. This cell line therefore constitutes a good model for studying the regulation of Duox2 expression in the colon.

NADPH oxidase 1 (NOX1) is another novel homolog of gp91phox identified in the human colon and in the colonic carcinoma cell line Caco-2 (27). NOX1 mRNA was recently found to be abundantly expressed in the epithelial cells of the mouse colon, with the greatest expression occurring predominantly in the first two-thirds of the crypt (12). The NOX1 transcript was induced in nonproliferating cells, suggesting that Nox1 performs specialized functions in the differentiated epithelial cells of the colon rather than having the mitogenic function previously proposed (27). It was found that the Nox1 protein was constitutively expressed in surface mucous cells of the guinea pig colon, and in primary culture, these cells spontaneously secreted superoxide anions, suggesting that Nox1 is constitutively active in this tissue (16). Cell-free assays revealed that Nox1 cannot generate O$_2^-$ per se (29). Substantial O$_2^-$ generation was only achieved in the presence of native neutrophil cytosol stimulated with PMA (29). p41$^{\text{Nox}}$ and p51$^{\text{Nox}}$, novel p47$^{\text{phox}}$ and p67$^{\text{phox}}$ homologs, were found to be essential for Nox1 to achieve potent oxidase activity (16). It was speculated that Nox1 could provide an oxidative barrier to defend the host against intestinal pathogens (16). Stimulation of Toll-like receptor (TLR)4 in guinea pig gastric mucosal cells by Helicobacter pylori LPS upregulated the Nox1 activity (15). Intestinal epithelial cells, T84 cells, specifically responded to rFliC and induced Nox1, although they were insensitive to LPS (16). It was speculated that stomach and colon may use different TLR members to recognize respective pathogenic microbes and activate Nox1.

Our findings show that Duox2 constitutes another source of ROS in the gut in addition to NOX1. Further studies are needed to identify the respective roles of the two oxidase systems in the epithelial cells of the colon and to explore their respective response to different stimulators.
ROS are implicated in the pathogenesis of the mucosal lesion in inflammatory bowel disease (IBD) (22), and their levels are elevated in the mucosa of patients as well as in experimental models of inflammation (17). Oxidative damage is a key contributor to a loss of barrier integrity and injury. Oxidation, which leads to the inhibition of essential protein function by inflammatory cells (neutrophils, macrophages, and lymphocytes), is a potential mechanism of tissue injury that may contribute to the pathogenesis of the disease. However, IBD is a disorder affecting all segments of the gut where Duox2, an H2O2-generating system, was found to be expressed. Disturbance of this system could therefore be implicated in the development of the inflammatory process. This work provides a starting point for investigating Duox2 as a new candidate mediator of physiopathological processes.

ACKNOWLEDGMENTS

We are indebted to Drs. Claudine Geffrotin and Silvia Vincent-Naulleau from Laboratoire Mixte CEA-INRA de Radiobiologie et d’Etude du Génome for collaboration and for assistance in tissue collection. Stanislas Morand is the recipient of a fellowship from the French Ministère de l’Education Nationale de la Recherche et de la Technologie. Sédami Gnidehou is the recipient of a fellowship from the French Embassy in Benin.

GRANTS

This work was supported in part by Bonus Qualité Recherche 2002, University of Paris XI.
REFERENCES


