Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 572 (2007) 74–81
www.elsevier.com/locate/ejphar

Hyperlipidemia induced by a cholesterol-rich diet aggravates necrotizing pancreatitis in rats

László Czakó [a,*], Annamária Szabolcs [a], Ágota Vajda [a], Sándor Csáti [a], Viktória Venglovecz [a], Zoltán Rakonczay Jr. [a], Péter Hegyi [a], László Tiszlavicz [b], Tamás Csont [c], Anikó Pósa [d], Anikó Berkó [d], Csaba Varga [d], Szőllősiné Varga Ilona [e], Imre Boros [f], János Lonovics [a]

[a] First Department of Medicine, University of Szeged, Hungary
[b] Department of Pathology, University of Szeged, Hungary
[c] Department of Biochemistry, University of Szeged, Hungary
[d] Department of Comparative Physiology, University of Szeged, Hungary
[e] Biological Isotope Laboratory, University of Szeged, Hungary
[f] Hungarian Academy of Sciences, Biological Research Center, Institute of Biochemistry, Szeged, Hungary

Received 19 February 2007; received in revised form 21 May 2007; accepted 23 May 2007
Available online 13 June 2007

Abstract

The aim of the present study was to investigate whether hyperlipidemia can cause acute pancreatitis or alter its severity. Male Wistar rats were fed a 3% cholesterol-enriched diet or a normal diet for 16 weeks. Edematous and necrotizing pancreatitis was induced with 3×75 μg/kg body weight of cholecystokinin s.c. and 2×2 g/kg body weight of L-arginine i.p., respectively, in separate groups of normal and hyperlipidemic rats. The severity of the pancreatitis was assessed. We studied the influence of hyperlipidemia on the formation of oxygen-derived free radicals, endogenous scavengers, nitric oxide synthases (NOS), peroxynitrite ($ONOO^-$), heat shock protein 72 (HSP72) and nuclear factor-kappa B (NF-κB) activation in the pancreas during acute edematous and necrotizing pancreatitis. Hyperlipidemia did not worsen edematous, but aggravated necrotizing pancreatitis. The cholesterol-enriched diet significantly reduced the catalase and Mn-superoxide dismutase (SOD) and constitutive NOS (cNOS) activities and increased the inducible NOS (iNOS) in the pancreas relative to those in the rats on the normal diet. The pancreatic nitrotyrosine level, as a marker of $ONOO^-$, and the NF-κB DNA-binding activity in the pancreas, were significantly elevated in the cholesterol-fed rats. The pancreatic HSP72 expression during necrotizing pancreatitis was not influenced by the hyperlipidemia. The pancreatic Mn-SOD, Cu, Zn-SOD, glutathione peroxidase, total glutathione and cNOS activities were significantly reduced, while the catalase, iNOS and NF-κB DNA-binding activities were significantly increased in the animals with necrotizing pancreatitis on the cholesterol diet as compared with those with pancreatitis and receiving the normal diet. Hyperlipidemia induced with this cholesterol-enriched diet leads to decreases in endogenous scavenger and cNOS activities, results in iNOS and NF-κB activation and stimulates $ONOO^-$ generation in the pancreas, which may be responsible for the aggravation of acute necrotizing pancreatitis.

Keywords: Hyperlipidemia; Acute edematous pancreatitis; Acute necrotizing pancreatitis; Peroxynitrite; NF-κB; Heat shock protein; Nitric oxide synthases; Endogenous scavengers

1. Introduction

Hyperlipidemia is reported to be associated with acute pancreatitis in 12–38% of the cases. Hyperlipidemia, which may lead to acute pancreatitis, may be seen as an epiphenomenon of pancreatitis. Lipid levels increase above the normal in up to

* Corresponding author. First Department of Medicine, University of Szeged, Szeged, P.O. Box: 427, H-6701, Hungary. Tel.: +36 62 545187; fax: +36 62 545185.
E-mail address: czal@in1st.szote.u-szeged.hu (L. Czakó).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.05.064

50% of patients with acute pancreatitis of any cause. The relationship between the two and the role of hyperlipidemia in the pathogenesis of acute pancreatitis is uncertain (Dominguez-Munoz et al., 1991; Toskes, 1990; Yadav and Pitchumoni, 2003).

Hyperlipidemia may be primary in origin or secondary to other clinical conditions, such as alcohol abuse, diabetes mellitus, pregnancy and the use of oral contraceptives. Consequently, most clinical reports have a high proportion of patients with alcoholism, which can itself induce acute pancreatitis. For ethical reasons, an experimental design that convincingly demonstrates causative or contributory effects of hyperlipidemia on acute pancreatitis is difficult to apply clinically. The role of hyperlipidemia in the pathogenesis of pancreatitis might therefore, not be deduced from clinical studies. It has been suggested that animal experiments should be resorted in order to assess the effect of hyperlipidemia on the course of acute pancreatitis (Zieve, 1968).

The mechanism of hyperlipidemic acute pancreatitis is not known. The increasing evidence that has accumulated in recent years indicates that a high-cholesterol diet impairs nitric oxide (NO)-cGMP signaling in both endothelial and nonendothelial cells (Ferdinandy et al., 1997; Deliconstantinos et al., 1995). In the normal pancreas, NO is synthesized from L-arginine (Arg) on the action of nitric oxide synthase (NOS), which exists in 3 isoforms: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutive (cNOS), and an inducible form (iNOS). NO appears to have a biphasic (protective and deleterious) role in acute pancreatitis (Vallance, 2003; Moncada and Higgs, 1993; Werner et al., 1998).

Experimental hypercholesterolemia is associated with an increased production of reactive oxygen species (ROS) (Parker et al., 1995), decreased activities of endogenous radical scavengers (Napoli et al., 1999), and a decreased bioavailability of NO (Ignarro et al., 1999). A reduced level of vascular NO release in hyperlipidemia has been revealed as a consequence of the enhanced formation of superoxide, which then reacts with NO to form the highly toxic peroxynitrite ion ($ONOO^-$) (White et al., 1994).

One of the most important transcription factors that control proinflammatory gene expression during acute pancreatitis is nuclear factor κB (NF-κB). In most cells, NF-κB is normally sequestered in the cytoplasm in an inactive form associated with a class of inhibitory proteins called IκBs. NF-κB is rapidly activated during acute pancreatitis, is translocated to the nucleus, binds to specific κB sequences in the promoter regions and transactivates the downstream genes, including interleukins, chemokines, adhesion molecules, receptors and enzymes (Barnes and Karin, 1997; Rakonczay et al., 2003a,b). Experimental hypercholesterinemia has been demonstrated to be associated with NF-κB activation in the coronary vasculature (Wilson et al., 2000). Moreover, NF-κB has been shown to play a critical role in the pathogenesis of acute experimental pancreatitis by regulating the expressions of many proinflammatory genes in the pancreas (Rakonczay et al., 2003a,b).

It is well known that the accumulation of the inducible member of the 70-kD heat shock protein family (HSP72) in response to a variety of stressors such as heat, mechanical stress, and ischemia confers long-lasting protection against further stress injury (Rakonczay et al., 2003a,b; Welch, 1993). Attenuation of HSP expression has been revealed in certain pathological conditions, such as aging, cardiac hypertrophy and hyperlipidemia (Csont et al., 2002; Locke and Tanguay, 1996; Tajima et al., 1997).

The aims of the present study were to investigate whether hyperlipidemia induced by a cholesterol-enriched diet can cause acute pancreatitis or alter its severity in rats and to analyze the possible pathomechanism. The effects of hyperlipidemia were examined on the levels of malondialdehyde (MDA), a marker of lipid peroxidation, endogenous scavengers and the various forms of NOS, on the generation of $ONOO^-$ and on the activation of NF-κB in the pancreas. A study was also made whether hyperlipidemia interacts with the pancreatic heat stress response.

2. Materials and methods

The experimental protocol followed the principles of Laboratory Animal Care of the National Institutes of Health, USA, and was approved by the ethics committee of the University of Szeged.

2.1. Animals and experimental protocol

80–100 g male Wistar rats were used. The animals were kept at a constant room temperature of 22±2 °C, under 12-h light–dark cycles, and were fed laboratory chow enriched with 3% cholesterol (cholesterol group) or standard chow (LATI, Gödöllő, Hungary) (control group) for 16 weeks. At the end of this 16-week controlled-diet period, acute edematous pancreatitis was induced with 3×75 μg/kg body weight of cholecystokinin (CCK) (Takács et al., 1996) s.c. (CCK and cholesterol+CCK groups), and acute necrotizing pancreatitis with 2×2 g/kg body weight of Arg i.p. (Czakó et al., 1998), in separate groups of normal and hyperlipidemic rats (Arg and cholesterol+Arg groups). The control rats received the same amount of 0.9% saline or an 8.6% solution of glycine in 0.9% saline at the same times instead of the CCK and Arg. At 6 h following the first CCK injection and at 24 h following the first Arg injection, the rats were sacrificed by aortic exsanguinations respectively, and the severity of the pancreatitis was assessed by measurement of the serum amylase and lipase concentrations, and the ratio pancreatic weight/body weight, and via the histology.

2.2. Serum assays

For serum assays, blood samples were centrifuged for 20 min at 2500×*g*. The serum amylase and lipase activities were determined by an Auto Analyzer (Prestige-24, Tokyo Boeki Medical System, Japan). Serum triglycerides and total cholesterol concentrations were measured in triplicates using commercially available colorimetric assay kits (Diagnosticum Rt, Budapest, Hungary) adapted to 96-well plates as described previously (Bjelik et al., 2006). The accuracy of the assays was monitored by using Standard Lipid Controls (Sentinel, Milan, Italy).

2.3. Redox status

The pancreata were homogenized in 4-fold excess (w/v) of ice-cold buffer containing 100 mM K_2HPO_4, 150 mM KCl, 100 mM EDTA, (pH=7.4), and 0.2% (w/v) butylated hydroxytoluene using an Ultra-Turrax homogenizer (IKA-Werk, Staufen, Germany) for 2 min. The homogenates were centrifuged at 3000 ×*g* for 10 min and the supernatants were used for measurements. MDA levels were measured after reaction with thiobarbituric acid, according to the method of Placer et al. (1966), and were corrected for the protein content of the tissue. Superoxide dismutase (SOD) activity was determined on the basis of the inhibition of epinephrine-adrenochrome autoxidation (Misra and Fridovich, 1972). Mn-SOD activity was measured by the autoxidation method in the presence of 5×10^{-3} M KCN (Beauchamp and Fridovich, 1971). Cu, Zn-SOD activity was calculated by subtracting the Mn-SOD activity from the overall SOD activity. Catalase activity was determined spectrophotometrically at 240 nm by the method of Beers et al. (Beers and Sizer, 1951) and was expressed in Bergmeyer units (BU) (1 BU=the decomposition of 1 g of H_2O_2/min at 25 °C). The total glutathione (GSH) content in the supernatant was measured spectrophotometrically with Ellman's reagent, and was corrected for the protein content of the tissue (Sedlak and Lindsay, 1968). Glutathione peroxidase activity was determined by the method of using cumene hydroperoxide and reduced glutathione as substrates of glutathion peroxidase (Chiu et al., 1976)).

2.4. Preparation of nuclear protein extracts and electrophoretic mobility shift assay (EMSA) of NF-κB

Preparation of nuclear protein extracts and EMSA was performed as described previously (Rakonczay et al., 2003a,b). Briefly, a 250–300-mg pancreatic tissue sample was lysed on ice in hypotonic *buffer A* by 20 strokes in a glass Dounce homogenizer. The hypotonic buffer was supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF), 4 mM benzamidine, 100 IU/ml aprotinin, and 1 mM dithiothreitol (DTT). The homogenate was left on ice for 25 min, and Nonidet P-40 was then added to a final concentration of 0.3–0.4% (v/v). The samples were briefly vortexed and incubated on ice for an additional 2 min. The nuclear pellet was collected by centrifugation of the lysed tissue for 50 s at 13,000 ×*g* in a microfuge. The supernatant (cytosolic fraction) was saved for Western blot analysis. The nuclear pellet was resuspended in *buffer C* supplemented with 1 mM DTT, 1.5 mM PMSF, 4 mM benzamidine, and 100 IU/ml aprotinin. After rotation at 4 °C for 30–45 min, the nuclear membranes were pelleted by microcentrifugation for 10 min and the supernatant (nuclear extract) was aliquoted and stored at −70 °C. For the EMSA of NF-κB DNA-binding activity, a 21-basepair oligonucleotide 5′-GGCAGAGGGGACTTTCCGAGA-3′ containing the NF-κB consensus sequence (underlined) was annealed with its complementary oligonucleotide (with 5′ G overhangs at both ends) to generate a double-stranded probe and was end-labeled with [γ-^{32}P] by T_4 polynucleotide kinase. To determine the NF-κB binding activity, aliquots of nuclear protein (15 μg) were mixed with a buffer containing 10 mM HEPES (pH=7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 4.5 μg poly(dI/dC). The binding reaction was started by adding 5–8000 cpm of the radiolabeled double-stranded probe and was allowed to proceed for 30–40 min on ice. The specificity of NF-κB binding was confirmed in competition experiments. DNA-protein complexes were resolved by PAGE at 4 °C on a nondenaturing 4.5% gel in a buffer containing 6.7 mM Tris base, 3.3 mM sodium acetate, and 1 mM EDTA (pH=7.5). Gels were vacuum-dried and exposed to Fuji RX films with intensifying screens at −70 °C. The intensities of the bands were quantified by using the ImageJ software (NIH, Bethesda, MD, USA).

2.5. Measurement of NOS

The activities of iNOS and cNOS were determined through the conversion of L-[^{14}C]arginine monohydrochloride to L-[^{14}C] citrulline (Takács et al., 2002). The protein concentration of the

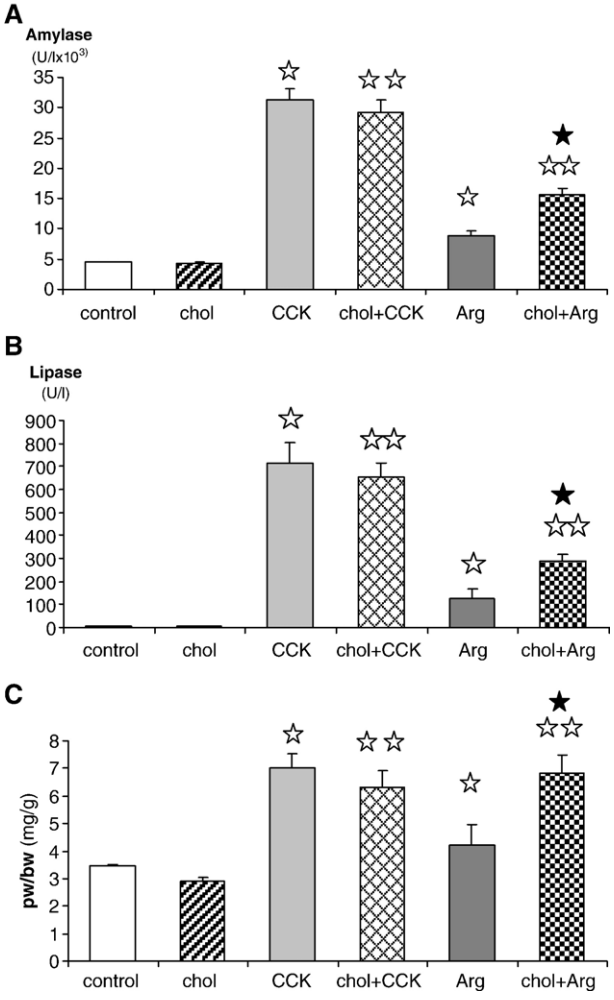

Fig. 1. Serum (A) amylase and (B) lipase activities, and (C) pancreatic edema expressed as the ratio pancreatic weight/body weight in the control, cholesterol-fed (chol) groups, and in normal and hyperlipidemic rats with edematous (CCK and chol+CCK) or necrotizing pancreatitis (Arg and chol+Arg). Results are means±S.E.M. (n=7). ☆: significant difference (P<0.05) vs. control group. ★: significant difference (P<0.05) vs. Arg group. ☆☆: significant difference (P<0.05) vs. chol group.

Table 1
Histological alterations in the control, cholesterol-fed (chol) groups, and in normal and hyperlipidemic rats with edematous (CCK and chol+CCK) and necrotizing pancreatitis (Arg and chol+Arg)

	Edema	Leukocyte infiltration	Acinar vacuolization	Hyperaemia	Necrosis	Total damage
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.20±0.20	0.00±0.00	0.20±0.20
chol	0.00±0.00	0.00±0.00	0.00±0.00	0.20±0.20	0.00±0.00	0.20±0.20
CCK	1.33±0.34 [a]	1.67±0.33 [a]	1.00±0.17 [a]	1.50±0.21 [a]	0.00±0.00	5.5±0.47 [a]
chol+CCK	1.33±0.34 [b]	1.33±0.29 [b, c]	0.66±0.28 [b]	1.67±0.18 [b; d]	0.00±0.00	4.99±0.42 [b]
Arg	1.50±0.34 [a]	1.67±0.33 [a]	1.17±0.17 [a]	1.67±0.21 [a]	1.14±0.14 [a]	7.15±0.65 [a]
chol+Arg	2.00±0.00 [b, d]	2.29±0.29 [b]	1.00±0.00 [b]	2.29±0.18 [b, d]	1.83±0.31 [b, d]	9.41±0.42 [b, d]

Results are means±S.E.M. (n=7).
[a] Significant difference (P<0.05) vs. the control group.
[b] Significant difference (P<0.05) vs. the chol group.
[c] Significant difference (P<0.05) vs. the CCK group.
[d] Significant difference (P<0.05) vs. the Arg group.

pancreatic tissue was determined by the method of Goa (Goa, 1953).

2.6. Western blotting

Western blot analysis of pancreatic HSP72, IκB-α expression and markers of $ONOO^-$ formation (by detecting 3-nitrotyrosine residues) was performed from the cytosolic fraction of the pancreas homogenate as described previously (Rakonczay et al., 2003a,b; Giricz et al., 2003). Pancreatic tissue was homogenized and diluted to load 20–40 μg of total protein on an 8–10% polyacrylamide gel. After separation by electrophoresis, the proteins were blotted onto nitrocellulose membrane. After blocking, the membranes were incubated with mouse monoclonal anti-nitrotyrosine antibody (Chemicon International, 1:1000 dilution, 80 min), rabbit anti-HSP72 (1:2500 dilution, 60 min), or rabbit anti-IκB-α (1:500 dilution, 60 min, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with a rabbit anti-mouse or goat anti-rabbit secondary antibody for 60 min (DakoCytomation Denmark A/S, Glostrup, Denmark, 1:1000). Bands were visualized by enhanced chemiluminescence (ECL Plus; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Thereafter, they were scanned and quantified by using the ImageJ software (NIH, Bethesda, MD, USA). The band densities of all 3-nitrotyrosine-containing proteins were determined and summed in order to estimate the total level of nitrated proteins. Results are expressed in arbitrary units.

2.7. Histologic examination

A portion of the pancreas was fixed overnight in 6% neutral formaldehyde solution and embedded in paraffin. Tissue slices were subjected to hematoxylin and eosin staining and histologic study by light microscopy. Slides were coded and examined blind by the pathologist for the grading of histologic alterations. Intestinal edema, vacuolization, inflammation, hemorrhage and acinar cell necrosis were graded on a scale of 1 to 3. The total histological damage was calculated by adding the scores for the different parameters.

2.8. Statistical analysis

Results are expressed as means±S.E.M. Experiments were evaluated statistically with two-way analysis of variance (ANOVA). P values<0.05 were accepted as statistically significant.

3. Results

3.1. Serum lipids

At the end of the 16-week controlled-diet period, the animals weighed 500–600 g. The hyperlipidemic rats were heavier, but not significantly so than the rats on the normal diet. The 16-week cholesterol-enriched diet significantly increased serum cholesterol and triglyceride levels from 1.88±0.15 and 0.52±0.05 mmol/L to

Table 2
The pancreatic activities of MDA and endogenous scavengers in the control, cholesterol-fed (chol) groups, and in normal and hyperlipidemic rats with necrotizing pancreatitis (Arg and chol+Arg)

	Malonyl dialdehyde (nM/mg protein)	Total glutathione (μM/mg protein)	Catalase (BU/mg protein×10^{-4})	Glutathion peroxidase (U/mg protein×10^{-3})	Mn-SOD (U/mg protein)	Cu, Zn-SOD (U/mg protein)
Control	0.10±0.005	1.01±0.12	1.7±0.14	4.97±0.25	0.81±0.06	2.63±0.15
Chol	0.12±0.01	1.08±0.18	1.1±0.10 [a]	5.92±0.34	0.64±0.05 [a]	2.55±0.20
Arg	3.21±0.18 [a]	1.71±0.14 [a]	6.51±0.51 [a]	12.07±0.63 [a]	0.31±0.03 [a]	5.28±0.33 [a]
Chol+Arg	3.42±0.20 [b]	1.24±0.11 [b, c]	8.74±0.73 [b, c]	9.1±0.60 [b, c]	0.20±0.02 [b, c]	4.01±0.26 [b, c]

Results are means±S.E.M. (n=7).
[a] Significant difference (P<0.05) vs. the control group.
[b] Significant difference (P<0.05) vs. the chol group.
[c] Significant difference (P<0.05) vs. the Arg group.

2.52±0.18 ($P<0.05$) and 1.07±0.12 ($P<0.05$) mmol/L, respectively.

3.2. Severity of acute pancreatitis

The cholesterol-enriched diet did not modify the serum amylase and lipase activities or the ratio pancreatic weight/body weight as compared with those of the rats on normal diet, and did not cause any histological alteration in the pancreas. Likewise, the cholesterol diet did not worsen the activities of serum amylase and lipase, the ratio pancreatic weight/body weight or the histological score in the animals with edematous pancreatitis. In marked contrast, in the animals with necrotizing pancreatitis, the serum amylase and lipase activities, the ratio pancreatic weight/body weight and the histological score were significantly increased in the hyperlipidemic animals as compared with the nonhyperlipidemic rats (Fig. 1, Table 1).

3.3. Oxidative stress

To analyze the mechanism by which hypercholesterinemia intensifies the course of acute necrotizing pancreatitis, we studied whether this high-cholesterol diet increased the extent of lipid peroxidation or the levels of endogenous scavengers due to oxidative stress in the pancreatic tissue. The pancreatic MDA concentration was not altered by the cholesterol diet in the rats without pancreatitis, while it was increased, but not significantly so in the hyperlipidemic group as compared with the nonhyperlipidemic rats with pancreatitis. Among the endogenous scavengers, the catalase and the Mn-SOD activities were significantly reduced following the cholesterol diet in the rats without pancreatitis. The Mn-SOD, Cu, Zn-SOD, glutathion peroxidase and GSH activities were significantly reduced, while the catalase activity was significantly increased in the hyperlipidemic pancreatitic animals as compared with the nonhyperlipidemic rats (Table 2).

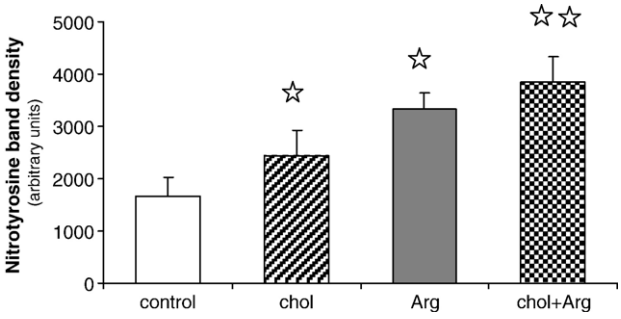

Fig. 3. Pancreatic nirotyrosine level, a marker for peroxynitrite generation. The bar chart show the band densities of all 3-nitrotyrosine-containing proteins. Rats were treated in the same manner as described in the legend to Fig. 2. Results are means±S.E.M. ($n=7$). ☆: significant difference ($P<0.05$) vs. control group. ☆☆: significant difference ($P<0.05$) vs. chol group.

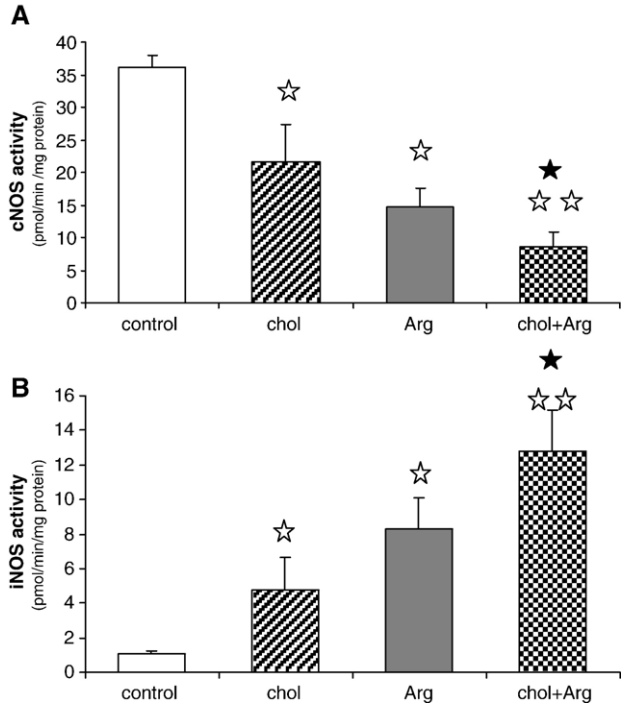

Fig. 2. Pancreatic (A) cNOS and (B) iNOS activity in the control, cholesterol-fed (chol) groups, and in normal and hyperlipidemic rats with necrotizing pancreatitis (Arg and chol+Arg). Results are means±S.E.M. ($n=7$). ☆: significant difference ($P<0.05$) vs. control group. ★: significant difference ($P<0.05$) vs. Arg group. ☆☆: significant difference ($P<0.05$) vs. chol group.

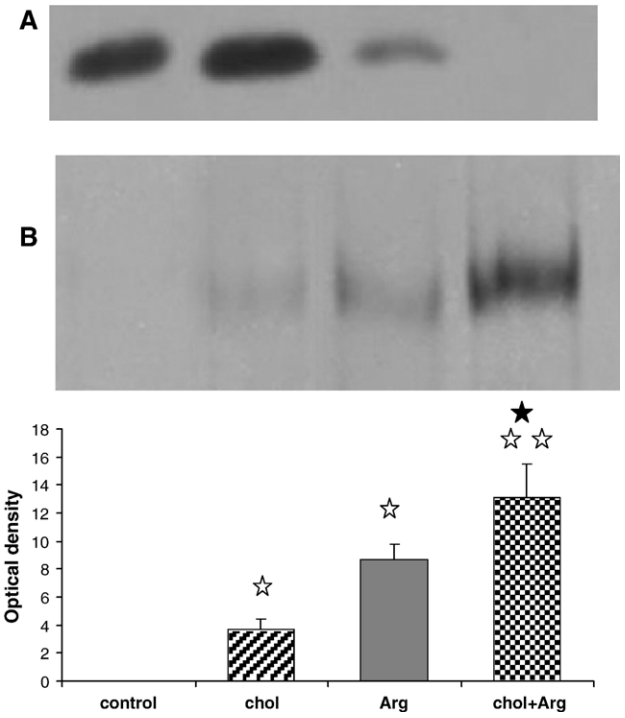

Fig. 4. Pancreatic IκB-α levels and NF-κB DNA-binding activity. (A) Pancreatic cytosolic protein fractions were analyzed by Western blot analysis (40 μg/lane), using a specific IκB-α antibody. (B) Pancreatic NF-κB DNA-binding activity was assessed by EMSA. The bar diagram shows the optical densities of the EMSA bands. Rats were treated in the same manner as described in the legend to Fig. 2. Results are means±S.E.M. ($n=7$). ☆: significant difference ($P<0.05$) vs. control group. ★: significant difference ($P<0.05$) vs. Arg group. ☆☆: significant difference ($P<0.05$) vs. chol group.

3.4. Pancreatic NO synthase

As concerns the activities of the free radical NO-synthesizing enzymes, the cNOS activity in the pancreas was significantly decreased, while that of iNOS was significantly increased as a result of the cholesterol diet. The cNOS activity was significantly further decreased in the animals with pancreatitis. Its level was significantly lower in the animals with pancreatitis on the cholesterol diet as compared with those on the normal diet. The iNOS activity was significantly increased in the animals with pancreatitis as compared with the rats without pancreatitis. The iNOS level was significantly higher in the animals with pancreatitis on the cholesterol diet as compared with those on the normal diet (Fig. 2).

3.5. Pancreatic $ONOO^-$ formation

High-cholesterol diet increased the formation of $ONOO^-$ in the pancreas, as the levels of pancreatic 3-nitrotyrosine (a marker of $ONOO^-$ formation) were found to be significantly increased in the cholesterol-fed rats as compared with the controls. The 3-nitrotyrosine level proved significantly higher in the animals with necrotizing pancreatitis (Fig. 3).

3.6. Pancreatic NF-κB activation

Interestingly, the cholesterol diet in itself led to significantly increased pancreatic NF-κB DNA-binding activity relative to the rats on the normal diet. Pancreatic IκB-α levels were not altered by cholesterol treatment. However, Arg administration significantly decreased IκB-α expression and this was further reduced in pancreatitic rats on a cholesterol diet. Furthermore, the level of NF-κB DNA-binding activity was significantly higher in the rats with necrotizing pancreatitis receiving the cholesterol diet as compared with the pancreatitic animals on the normal diet (Fig. 4).

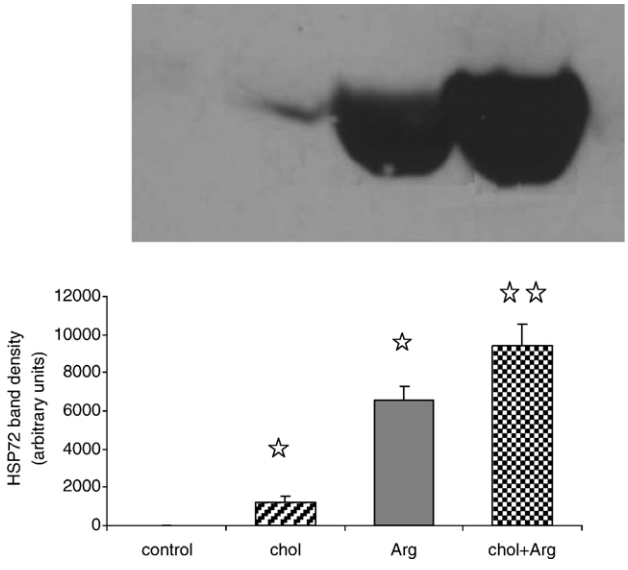

Fig. 5. Pancreatic HSP72 expression as assessed by Western blotting. The densities of the Western blot bands were quantified by using the ImageJ software. Rats were treated in the same manner as described in the legend to Fig. 2. Results are means ± S.E.M. ($n=7$). ☆: significant difference ($P<0.05$) vs. control group. ☆☆: significant difference ($P<0.05$) vs. chol group.

3.7. Pancreatic HSP72 protein expression

We assessed if hyperlipidemia induced by cholesterol-enriched diet affected the production of HSP72 in the pancreas in response to necrotizing pancreatitis. In the pancreas of the control rats, the basal level of HSP72 was very low, but the cholesterol-enriched diet significantly increased its expression. Arg-induced necrotizing pancreatitis resulted in further significant increases in pancreatic HSP72 content both in the animals on the normal diet and also in those on the cholesterol diet as compared with the controls (Fig. 5).

4. Discussion

The present results show that the rats on this cholesterol-enriched diet for 16 weeks exhibited reduced endogenous scavengers and cNOS activities and increased iNOS and NF-κB DNA binding activities, enhanced $ONOO^-$ formation in the pancreas and aggravation of their necrotizing pancreatitis.

A hyperlipidemia prevalence of 12–38% has been reported in acute human pancreatitis in previous studies. This wide range of hyperlipidemia in acute pancreatitis seems to result from the variations in the patient population, since alcohol consumption and diabetes mellitus may themselves cause hyperlipidemia (Dominguez-Munoz et al., 1991; Toskes, 1990; Yadav and Pitchumoni, 2003). Accordingly, we have to rely on animal studies to evaluate hyperlipidemia as a risk factor in acute pancreatitis. Only a few animal studies have been published, but the results are contradictory. In isolated *ex-vivo* perfused dog pancreata, hyperlipidemia was found to induce histological and serological alterations of acute pancreatitis (Saharia et al., 1977). No confirmatory studies have been reported. The contributory effect of hyperlipidemia has also been demonstrated. Endogenous hyperlipidemia was observed to intensify the course of acute edematous and necrotizing pancreatitis in the rat (Hofbauer et al., 1996), while exogenous triglycerides increased the pancreatic damage in acute edematous and necrotizing pancreatitis, initiated via different pathogenetic pathways in the isolated perfused pancreas (Kimura and Mossner, 1996). However, other reports suggest that hyperlipidemia does not aggravate the course of acute edematous pancreatitis in rats (Paye et al., 1995, 1996). The role of hyperlipidemia in acute pancreatitis therefore, seems questionable.

The present study demonstrated that this high-cholesterol diet in itself did not damage the exocrine pancreas, and did not alter the course of acute edematous pancreatitis, but it did aggravate acute necrotizing pancreatitis. The discrepancies between our findings and those in the previous studies may be explained by methodological differences (Saharia et al., 1977; Hofbauer et al., 1996; Kimura and Mossner, 1996). All of the previous animal models involved studies of the effects of acute hyperlipidemia induced by triglyceride infusion or by the injection of an active detergent (Triton WR 1339) leading to

endogenous hyperlipidemia. However, in clinical practice patients usually present with long-standing hyperlipidemia. We therefore, applied hyperlipidemia induced by a cholesterol-enriched diet, which better resembles the human situation.

The catalase and Mn-SOD activities were significantly reduced in the pancreas following the cholesterol diet. Accumulating evidence indicates that oxidative stress in the arterial wall plays a major role in the initiation and progression of the cardiovascular dysfunction associated with hyperlipidemia (Taniyama and Griendling, 2003). This is the first demonstration that a high-cholesterol diet leads to reduced levels of endogenous scavengers in the pancreas. Oxidative stress is a state in which excess ROS overwhelm endogenous antioxidant systems. One of the most important ROS in the vasculature is the superoxide radical anion (O_2^-), formed by the one-electron reduction of the oxygen molecule. SOD transforms O_2^- to the more stable hydrogen peroxide (H_2O_2), which is then converted enzymatically into H_2O by catalase and glutathion peroxidase. Hyperlipidemia reduces Mn-SOD, which may lead to the diminished elimination of O_2^-. Further, the prophylactic administration of a scavenger prior to the induction of acute pancreatitis exerts a beneficial effect on the development of pancreatitis (Czakó et al., 1998; Araki et al., 2003). It is therefore plausible to speculate that the reduced scavenger activity makes the pancreas more vulnerable to further stress, such as that of acute pancreatitis, which may result in more severe damage. Indeed, the activities of GSH, glutathion peroxidase, Mn-SOD and Cu, Zn-SOD were all significantly depleted in the rats with necrotizing pancreatitis receiving the high-cholesterol diet as compared with those on the normal diet.

Besides the reduced endogenous scavenger activities, we observed decreased cNOS and increased iNOS activities in the pancreas following the cholesterol diet. NO appears to have a biphasic (protective and deleterious) effect in acute pancreatitis. A small amount of NO derived from cNOS accounts for the protective action through the regulation of various housekeeping functions, while a large amount of NO derived from iNOS, induced by inflammatory cytokines and endotoxins, mediates the deleterious action through cytotoxic action (Vallance, 2003; Moncada and Higgs, 1993; Werner et al., 1998; Takács et al., 2002). It also emerged that the pancreatic iNOS activity was significantly higher and the cNOS activity was significantly lower in the rats with pancreatitis on the high-cholesterol diet as compared with those in the rats on the normal diet. This imbalance of the NO pathway may be responsible for the more severe pancreatitis seen in the hyperlipidemic rats.

It is well known that NO reacts rapidly with O_2^- to form $ONOO^-$, a potentially deleterious ROS. Hyperlipidemia has been shown to enhance the production of $ONOO^-$ in the vasculature and the heart (Beckman and Koppenol, 1996). The present study demonstrated that the high-cholesterol diet increased the pancreatic level of nitrotyrosine, a marker of $ONOO^-$ generation. The cytotoxic effects of $ONOO^-$ include lipid peroxidation, the nitration of tyrosine residues, the oxidation of sulfhydryl groups, DNA-strand breakage, and the inhibition of mitochondrial respiration, leading to tissue injury (Beckman and Koppenol, 1996). $ONOO^-$ generation in the pancreas of the hyperlipidemic rats may have contributed to the more severe pancreatitis seen in the rats on the high-cholesterol diet.

The NF-κB DNA-binding activity was significantly increased in the pancreas following the cholesterol diet. Unexpectedly, pancreatic IκB-α levels were unaltered by cholesterol treatment. It is possible that the cholesterol-induced NF-κB activation is regulated by IκB-β. NF-κB activation has been related to NO bioavailability and ROS production: while ROS contribute to NF-κB activation, an intact NO pathway system stabilizes it and prevents its activation. Thus, a balance between the oxidative status and the NO-dependent pathways may be one of the regulatory mechanisms of NF-κB activation (Li and Karin, 1999; Peng et al., 1995). In our study, the hyperlipidemia activated both the ROS and the NO pathway systems, and consequently both may contribute to NF-κB activation. The NF-κB DNA-binding activity was significantly higher and IκB-α levels were significantly lower in the rats with necrotizing pancreatitis receiving the high-cholesterol diet as compared with those on the normal diet. The increased activation of NF-κB may be responsible in part for the more severe pancreatitis in the hyperlipidemic rats by activating the many proinflammatory genes in the pancreas.

Hyperlipidemia has been shown to attenuate heat shock protein expression in the heart (Csont et al., 2002). Although, it was not known whether hyperlipidemia leads to a decreased heat shock response in the pancreas, it was tempting to speculate that this mechanism is involved in the increased severity of pancreatitis in hyperlipidemia. Accordingly, we measured the pancreatic HSP72 production. Pancreatic HSP72 was induced by acute necrotizing pancreatitis in animals on the high-cholesterol diet and in others on the normal diet; there was no significant difference in HSP72 expression between the two groups.

The present study involved an *in vivo* model in which the direct effect of plasma triglycerides cannot be excluded. Pancreatic lipase breaks down triglycerides to free fatty acids. Free fatty acids are toxic: they may damage acinar cells directly and injure the vascular endothelium, leading to disturbances of the microcirculation. Moreover, trypsinogen may be activated by acidosis due to the presence of free fatty acids (Saharia et al., 1977; Havel, 1969; Niederau and Grendell, 1998). These mechanisms can also take part in the development of hyperlipidemic pancreatitis.

In summary, the present study revealed that hyperlipidemia decreases the endogenous free radical scavengers and cNOS activities, induces iNOS and NF-κB activation and stimulates $ONOO^-$ generation in the pancreas, which may be responsible for the aggravation of acute necrotizing pancreatitis. Targeting these inflammatory mediators with pharmacological tools could possibly form the basis of a new strategy with which to treat or prevent acute pancreatitis aggravated by hyperlipidemia.

Acknowledgments

This work was supported in part by grants from the Hungarian National Research Fund (OTKA no. T049134), the Hungarian Academy of Sciences (BÖ 5/2003) and the Medical Research Council (ETT 499/2006). The anti-HSP72 antibody was a generous gift from István Kurucz (IVAX Drug Research Institute, Budapest, Hungary).

References

Araki, Y., Andoh, A., Yokono, T., Asano, N., Yoshikawa, K., Bamba, S., Ishizuka, I., Fujiyama, Y., 2003. The free radical scavenger edaravone suppresses experimental closed duodenal loop-induced acute pancreatitis in rats. Int. J. Mol. Med 12, 121–124.

Barnes, P.J., Karin, M., 1997. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med 336, 1066–1071.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. Anal. Biochem 44, 276–287.

Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol 271, C1424–C1437.

Beers Jr., R.F., Sizer, I.W., 1951. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem 195, 133–140.

Bjelik, A., Bereczki, E., Gonda, S., Juhász, A., Rimanóczy, A., Zana, M., Csont, T., Pakaski, M., Boda, K., Ferdinandy, P., Dux, L., Janka, Z., Sántha, M., Kálmán, J., 2006. Human apoB overexpression and a high-cholesterol diet differently modify the brain APP metabolism in the transgenic mouse model of atherosclerosis. Neurochem. Int 49, 393–400.

Chiu, D.T., Stults, F.H., Tappel, A.L., 1976. Purification and properties of rat lung soluble glutathione peroxidase. Biochim. Biophys. Acta 445, 558–566.

Csont, T., Balogh, G., Csonka, C., Boros, I., Horváth, I., Vigh, L., Ferdinandy, P., 2002. Hyperlipidemia induced by high cholesterol diet inhibits heat shock response in rat hearts. Biochem. Biophys. Res. Commun. 290, 1535–1538.

Czakó, L., Takács, T., Varga, I.S., Tiszlavicz, L., Hai, D.Q., Hegyi, P., Matkovics, B., Lonovics, J., 1998. Involvement of oxygen-derived free radicals in L-arginine-induced acute pancreatitis. Dig. Dis. Sci. 43, 1770–1777.

Deliconstantinos, G., Villiotou, V., Stavrides, J.C., 1995. Modulation of particulate nitric oxide synthase activity and peroxynitrite synthesis in cholesterol enriched endothelial cell membranes. Biochem. Pharmacol. 49, 1589–1600.

Dominguez-Munoz, J.E., Malfertheiner, P., Ditschuneit, H.H., Blanco-Chavez, J., Uhl, W., Buchler, M., Ditschuneit, H., 1991. Hyperlipidemia in acute pancreatitis. Relationship with etiology, onset, and severity of the disease. Int. J. Pancreatol. 10, 261–267.

Ferdinandy, P., Szilvássy, Z., Horváth, L.I., Csont, T., Csonka, C., Nagy, E., Szentgyörgyi, E., Nagy, I., Koltai, M., Dux, L., 1997. Loss of pacing-induced preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. J. Mol. Cell Cardiol. 29, 3321–3333.

Giricz, Z., Csonka, C., Onody, A., Csont, T., Ferdinandy, P., 2003. Role of cholesterol-enriched diet and the mevalonate pathway in cardiac nitric oxide synthesis. Basic Res. Cardiol. 98, 304–310.

Goa, J., 1953. A micro biuret method for protein determination; determination of total protein in cerebrospinal fluid. Scand. J. Clin. Lab. Invest. 5, 218–222.

Havel, R.J., 1969. Pathogenesis, differentiation and management of hypertriglyceridemia. Adv. Intern. Med. 15, 117–154.

Hofbauer, B., Friess, H., Weber, A., Baczako, K., Kisling, P., Schilling, M., Uhl, W., Dervenis, C., Buchler, M.W., 1996. Hyperlipaemia intensifies the course of acute oedematous and acute necrotising pancreatitis in the rat. Gut 38, 753–758.

Ignarro, L.J., Cirino, G., Napoli, C., 1999. Nitric oxide as a signaling molecule in the vascular system: an overview. J. Cardiovasc. Pharmacol. 1, 879–886.

Kimura, W., Mossner, J., 1996. Role of hypertriglyceridemia in the pathogenesis of experimental acute pancreatitis in rats. Int. J. Pancreatol. 20, 177–184.

Li, N., Karin, M., 1999. Is NF-kappaB the sensor of oxidative stress? FASEB J. 13, 1137–1143.

Locke, M., Tanguay, R.M., 1996. Diminished heat shock response in the aged myocardium. Cell Stress Chaperones 1, 251–260.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Moncada, S., Higgs, A., 1993. The L-arginine-nitric oxide pathway. N. Engl. J. Med. 329, 2002–2012.

Napoli, C., Witztum, J.L., de Nigris, F., Palumbo, G., D'Armiento, F.P., Palinski, W., 1999. Intracranial arteries of human fetuses are more resistant to hypercholesterolemia-induced fatty streak formation than extracranial arteries. Circulation 99, 2003–2010.

Niederau, C., Grendell, J.H., 1998. Intracellular vacuoles in experimental acute pancreatitis in rats and mice are an acidified compartment. J. Clin. Invest. 81, 229–236.

Parker, R.A., Sabrah, T., Cap, M., Gill, B.T., 1995. Relation of vascular oxidative stress, alpha-tocopherol, and hypercholesterolemia to early atherosclerosis in hamsters. Arterioscler. Thromb. Vasc. Biol. 15, 349–358.

Paye, F., Chariot, J., Molas, G., Benessiano, J., Roze, C., 1995. Nonesterified fatty acids in acute cerulein-induced pancreatitis in the rat. Are they really deleterious in vivo? Dig. Dis. Sci. 40, 540–545.

Paye, F., Chariot, J., Molas, G., Benessiano, J., Roze, C., 1996. Release of nonesterified fatty acids during cerulein-induced pancreatitis in rats. Dig. Dis. Sci. 41, 1959–1965.

Peng, H.B., Libby, P., Liao, J.K., 1995. Induction and stabilisation of IkBalpha by nitric oxide mediates inhibition of NF-κB. J. Biol. Chem. 270, 14214–14219.

Placer, Z.A., Cushman, L., Johnson, B.C., 1966. Estimation of product of lipid peroxidation (malonyl dialdehydes) in biochemical systems. Anal. Biochem. 16, 359–364.

Rakonczay Jr., Z., Jármay, K., Kaszaki, J., Mándi, Y., Duda, E., Hegyi, P., Boros, I., Lonovics, J., Takács, T., 2003a. NF-κB activation is detrimental in arginine-induced acute pancreatitis. Free Radic. Biol. Med. 34, 696–709.

Rakonczay Jr., Z., Takács, T., Boros, I., Lonovics, J., 2003b. Heat shock proteins and the pancreas. J. Cell Physiol 195, 383–391.

Saharia, P., Margolis, S., Zuidema, G.D., Cameron, J.L., 1977. Acute pancreatitis with hyperlipemia: studies with an isolated perfused canine pancreas. Surgery 82, 60–67.

Sedlak, J., Lindsay, R.H., 1968. Estimation of total, protein-bound, and nonprotein sulfhydryls groups in tissue with Ellman's reagent. Anal. Biochem. 25, 192–205.

Tajima, M., Isoyama, S., Nitta, Y., Abe, K., 1997. Attenuation of heat shock protein expression by coronary occlusion in hypertrophied hearts. Am. J. Physiol. 273, H526–H533.

Takács, T., Farkas Jr., Gy., Czakó, L., Jármay, K., Mándi, Y., Lonovics, J., 1996. Time-course changes in serum cytokine levels in two experimental acute pancreatitis models in rats. Res. Exp. Med. 196, 153–161.

Takács, T., Czakó, L., Morschl, É., László, F., Tiszlavicz, L., Rakonczay Jr., Z., Lonovics, J., 2002. The role of nitric oxide in edema formation in L-arginine-induced acute pancreatitis. Pancreas 25, 277–282.

Taniyama, Y., Griendling, K.K., 2003. Reactive oxygen species in the vasculature. Molecular and cellular mechanisms. Hypertension 42, 1075–1081.

Toskes, P.P., 1990. Hyperlipidemic pancreatitis. Gastroenterol. Clin. North. Am. 19, 783–791.

Vallance, P., 2003. Nitric oxide: therapeutic opportunities. Fundam. Clin. Pharmacol. 17, 1–10.

Welch, W.J., 1993. Heat shock proteins functioning as molecular chaperones: their roles in normal and stressed cells. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 339, 327–333.

Werner, J., Fernandez-del Castillo, C., Rivera, J.A., Kollias, N., Lewandrowski, K.B., Rattner, Q.W., Warshaw, A.L., 1998. On the protective mechanisms of NO in acute pancreatitis. Gut 43, 401–407.

White, C.R., Brock, T.A., Chang, L.Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W.A., Gianturco, S.H., Gore, J., Freeman, B.A., Tarpey, M.M., 1994. Superoxide and peroxynitrite in atherosclerosis. Proc. Natl. Acad. Sci. U. S. A. 91, 1044–1048.

Wilson, S.H., Caplice, N.M., Simari, R.D., Holmes Jr., D.R., Carlson, P.J., Lerman, A., 2000. Activated nuclear factor-kappa B is present in the coronary vasculature in experimental hypercholesterolemia. Atherosclerosis 148, 23–30.

Yadav, D., Pitchumoni, C.S., 2003. Issues in hyperlipidemic pancreatitis. J. Clin. Gastroenterol. 36, 54–62.

Zieve, L., 1968. Relationship between acute pancreatitis and hyperlipemia. Med. Clin. North Am. 52, 1493–1501.