Neutral Sphingomyelinase Action Stimulates Signal Transduction of Tumor Necrosis Factor-α in the Synthesis of Cholesteryl Esters in Human Fibroblasts*

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We have investigated biochemical mechanisms of tumor necrosis factor (TNF-α) signaling in cultured human skin fibroblasts. We found that TNF-α signaling may involve activation of a cell membrane neutral sphingomyelinase (N-SMase) in that within 2.5–5 min of treatment of cells with TNF-α there was a 2-fold increase in the activity of N-SMase compared to control. This reaction led to the hydrolysis of sphingomyelin as evidenced by a decrease in sphingomyelin mass and in the radioactivity associated with [14C]cholesterol-labeled sphingomyelin. This was accompanied by a 4-fold increase in the formation of cholesteryl [14C]oleate within 2.5 min of incubation with TNF-α. This reaction also stimulated the mobilization of cell surface-associated [3H]cholesterol and its utilization in the synthesis of [3H]cholesterol esters via acyl coenzyme-A cholesterol acyltransferase (ACAT). Gas chromatographic analysis revealed that the cellular level of cholesteryl esters increased about 2.5–3-fold following treatment with TNF-α compared to control. Cholesteryl ester synthesis was compromised upon incubation of cells with antibody against N-SMase and remained unaltered with TNF-β and fibroblast growth factor. Furthermore, TNF-α-mediated stimulation of cholesteryl ester synthesis was compromised by incubation of cells with an inhibitor of ACAT. These findings suggest a possible biological role of N-SMase in the signal transduction of TNF-α in the synthesis of cholesteryl esters in human fibroblasts.

Human neutral sphingomyelinase (N-SMase) is a cell surface-associated enzyme that contributes to the catabolism of sphingomyelin to ceramide and phosphocholine. It is accompanied by decreased radioactivity associated with sphingomyelin and increased synthesis of cholesteryl esters derived from cell membrane cholesterol.

MATERIALS AND METHODS

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The abbreviations used are: N-SMase, neutral sphingomyelinase; TNF-α, tumor necrosis factor; ACAT, acyl-CoA-cholesterol fatty acyltransferase; EC 1.1.1.88; FGF, fibroblast growth factor.
incubation with TNF-α. Thereafter, the activity of N-SMase did not increase further. In contrast, TNF-B (50 units/ml medium) did not alter the activity of N-SMase in fibroblasts (data not shown).

When cells were prelabeled with [14C]choline, washed, and incubated with TNF-α, within 2.5 min we observed a 30% decrease in the radioactivity incorporated into sphingomyelin compared with control (Fig. 1B). Incubation of cells with TNF-α up to 60 min further decreased the incorporation of [14C]choline into sphingomyelin on the order of 40% compared with control. TNF-α concurrently and consistently decreased the cellular levels of sphingomyelin on the order of 30% over the 2.5–60 min period compared to control (Fig. 1C). For example, cells treated without TNF-α contained 70 μg of sphingomyelin/mg protein. Upon incubation with TNF-α cellular levels of sphingomyelin was on the order of 49–50 μg/mg protein. The cellular levels of phosphatidylcholine in control cells was on the order of 140 μg/mg protein. This value did not change significantly upon incubation of cells with TNF-α.

**Effects of Time of Incubation with TNF-α on the Synthesis of Cholesteryl Ester from Exogenous [14C]Oleate**—We observed a marked increase in the formation of cholesterol oleate (derived from exogenous [14C]oleate) within 2.5–5 min of incubation of cells with TNF-α (Fig. 2). In that, we observed a 4-fold increase in the formation of cholesterol [14C]oleate during this time period in cells incubated with TNF-α compared to control. Incubation of cells with TNF-α for 30–60 min did not significantly increase the formation of cholesterol [14C]oleate further (Fig. 2).

**Effects of Time of Incubation with TNF-α on the Utilization of Cell Surface Cholesterol for [3H]Cholesteryl Ester Synthesis**—As shown in Fig. 3, the synthesis of cholesteryl esters from plasma membrane-derived [3H]cholesterol in control fibroblasts over a period of 60 min was insignificant. In contrast, in cells incubated with TNF-α (10 units/ml) there was a significant time-dependent increase in the utilization of plasma membrane-derived [3H]cholesterol for the synthesis of [3H]cholesteryl esters. Within 2.5 min of incubation of cells with TNF-α there was a rapid increase in the synthesis of cholesteryl esters from cell membrane derived [3H]cholesterol. Thereafter, there was a modest increase in cholesteryl ester synthesis in cells incubated with TNF-α up to 60 min.

**Effects of TNF-α Concentration on the Activity of N-SMase in Fibroblasts**—As shown in Fig. 4, TNF-α exerted a concentration-dependent increase in the activity of N-SMase in fibroblasts. The maximum increase in N-SMase activity (a 2.7-fold increase compared with control) occurred upon incubation of cells with 10 units (60 ng) of TNF-α/ml medium. At higher concentrations of TNF-α (25–50 units/ml medium), there was only a 2-fold increase in N-SMase activity compared with control.
Effects of TNF-α, TNF-β, and FGF (acidic) Concentration on the Synthesis of Cholesteryl Oleate—In human skin fibroblasts, TNF-α increased the synthesis of cholesteryl oleate as a function of concentration (Fig. 5) of incubation. A maximum of 5-fold increase in cholesteryl oleate synthesis compared with control occurred upon incubating cells with 50 units (300 ng) of TNF-α/ml medium for 1 h at 37 °C (Fig. 5). In contrast, TNF-β and FGF did not alter the synthesis of cholesteryl oleate in these cells (Fig. 5).

Effects of TNF-α Concentration on the Cellular Level of Cholesteryl Esters—As shown in Fig. 6, in normal human fibroblasts TNF-α exerted a concentration-dependent increase in the mass of cholesteryl esters. Maximum increase in cholesteryl ester levels (2.7-fold higher than control) occurred upon incubation with 25 units (150 ng) of TNF-α/ml medium. Incubation of cells with higher concentration of TNF-α did not increase the mass of cholesteryl ester in cells, further.

Effects of ACAT Inhibitor and Co-incubation with N-SMase and TNF-α on the Synthesis of Cholesteryl Esters—Incubation of cells with N-SMase (0.1 μg/ml) and with TNF-α (30 ng/ml medium) increased the synthesis of cholesteryl oleate on the order of 3.93 ± 0.2- and 1.56 ± 0.02-fold, respectively, compared with control. Incubation of cells with a mixture of N-SMase and TNF-α stimulated cholesteryl ester synthesis in an additive fashion: the increase in cholesteryl oleate synthesis was on the order of 4.9 ± 0.4-fold. However, ACAT inhibitor alone and ACAT inhibitor plus TNF-α compromised the stimulatory effects of TNF-α on the formation of cholesteryl-[14C]oleate on the order of 0.15 ± 0.05- and 0.72 ± 0.10-fold, respectively, as compared with control.

DISCUSSION

The major findings of this study are summarized in the hypothetical model in Fig. 7. First, exogenous TNF-α increases the endogenous activity of N-SMase in human fibroblasts. Second, cell membrane sphingomyelin is hydrolyzed to ceramide and phosphocholine due to N-SMase action. Third, the depletion of cell membrane sphingomyelin may lead to the mobilization of cell membrane cholesterol. Fourth, the membrane-derived cholesterol is converted to cholesteryl esters via ACAT.

Previous studies have shown that sphingomyelin has a high affinity for cholesterol and keeps cholesterol in the plasma membrane compartment of mammalian cells (1). Previous studies have also revealed a strong relationship between N-SMase action and cholesteryl ester synthesis (8, 9, 16) in that within minutes of addition of exogenous N-SMase there was a rapid increase in the utilization of membrane bound [3H]cholesterol for the synthesis of cholesteryl esters. We have tested this relationship in greater detail employing TNF-α in our
studies. Human skin fibroblasts have an approximately equal amount of cholesterol and sphingomyelin (70 μg/mg protein), and TNF-α and N-SMase can decrease the level of sphingomyelin on the order of 30% (as shown in Fig. 1C) and up to 50%, respectively (9). Thus, about 21–35 μg of sphingomyelin may be hydrolyzed upon treatment of cells with TNF-α or N-SMase, respectively. Assuming that an approximately equal amount of cholesterol was removed from the cell surface, then almost 10 μg was used for cholesteryl ester synthesis (Fig. 6). Such findings are consistent with previously published data (9, 16) in which exogenous sphingomyelinase was also shown to decrease cellular levels of sphingomyelin and stimulate cholesteryl ester synthesis in an approximately proportional fashion. Moreover, when we prelabeled fibroblasts with [3H]cholesterol, that has been previously shown to be esterified by ACAT (16), and incubated such cells with TNF-α, within 2.5 min (when almost maximum stimulation of N-SMase had occurred) (Fig. 1A), we observed a marked increase in [3H]cholesteryl ester formation (Fig. 3). During this time period, we also observed a 4-fold increase in the incorporation of [14C]oleate into cholesteryl [14C] oleate. Taken together, our studies indicate that there is no lag in the stimulation of cholesteryl ester synthesis following TNF-α-mediated activation of N-SMase. Rather, cell surface cholesterol is rapidly utilized to synthesize cholesteryl esters.

Other studies have shown that cholesterol released from the cell surface due to N-SMase-mediated depletion of sphingomyelin levels is not egressed in the presence of high density lipoproteins in the cultured medium (18). Rather, it is destined for esterification via ACAT. This tenet is further supported by our finding that ACAT inhibitor could compromise the stimulatory effect of TNF-α. Previously, a dramatic increase in the activity of ACAT was reported in fibroblasts incubated with exogenous N-SMase (16). Thus, the cell surface cholesterol is utilized for cholesteryl ester synthesis via ACAT. However, we and others have previously showed that the catabolic products of sphingomyelin, e.g. ceramide, phosphocholine, and sphingosine, have no effects on cholesteryl ester synthesis (9, 18).

Our studies reveal that antibody against N-SMase (2) compromised the stimulatory effects of TNF-α on cholesteryl ester synthesis. We have previously shown that antibody against N-SMase does not alter cell viability (9). Moreover, incubation of cells with exogenous N-SMase plus corresponding antibody compromised the stimulatory effects of N-SMase on low density lipoprotein receptor activity and cholesteryl ester synthesis (9). Whereas, preimmune serum IgG did not alter this reaction. Studies from our laboratory in human PT cells (19) and in human skin fibroblasts (20) reveal that N-SMase is localized on the surface of these cells. Such topology may provide TNF-α direct access to N-SMase. However, recently Shutze et al. (20) have suggested that TNF-α-mediated sphingomyelin breakdown may be carried out by an acidic sphingomyelinase. On the other hand, the cytosolic origin of N-SMase in HL-60 cells has been suggested.3 Clearly, studies are required to address this issue further.

In summary, our findings indicate that by controlling the microenvironnent of the cell surface in which cholesterol and sphingomyelin reside, biomodulators (e.g. TNF-α) but not TNF-β and FGF may facilitate the regulation of cholesterol and sphingomyelin metabolism. This is an important finding as it suggests a possible biological role of cell surface N-SMase in the signal transduction of TNF-α in normal human fibroblasts. Further studies will be required to explain the detailed biochemical mechanisms involved in this process. Such studies are underway in our laboratory.

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