α-Lipoic Acid as a Directly Binding Activator of the Insulin Receptor: Protection from Hepatocyte Apoptosis†

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Received December 12, 2006

ABSTRACT: Background and aim: α-Lipoic acid has cytoprotective potential which has previously been explained by its antioxidant properties. The aim of this study was to assess LA-induced-specific cytoprotective signalling pathways in hepatocytes. Methods: Apoptosis of rat hepatocytes was induced by actinomycinD/TNF-α. Caspase-3-like activity was determined by a fluorometric; LDH by an enzymatic assay; and phosphorylation of the insulin receptor, Akt, and Bad by Western blot (after immunoprecipitation). Protein kinase and insulin receptor activities were measured by in vitro phosphorylation. Computer modeling studies were performed by using the program GRID. Results: α-Lipoic acid decreased actinomycinD/TNF-α-induced apoptosis, as did the antioxidants Trolox and *N*-acetylcysteine. The activation of PI3-kinase/Akt involving phosphorlyation of Bad markedly contributed to the cytoprotective action of α-lipoic acid. α-Lipoic acid but not other antioxidants protected against actinomycinD/TNF-α-induced apoptosis via phosphorylation of the insulin receptor. Computer modeling studies revealed a direct binding site for α-lipoic acid at the tyrosine kinase domain of the insulin receptor, suggesting a stabilizing function in loop A that is involved in ATP binding. Treatment of immunoprecipitated insulin receptor with LA induced substrate phosphorylation. Conclusions: α-Lipoic acid mediates its antiapoptotic action via activation of the insulin receptor/PI3-kinase/Akt pathway. We show for the first time a direct binding site for α-lipoic acid at the insulin receptor tyrosine kinase domain, which might make α-lipoic acid a model substance for the development of insulin mimetics.

α-Lipoic acid (LA[1]; 1,2-dithiocyclopentan-3-valeric acid, thioctic acid) is a biogenic antioxidant that physiologically acts as a coenzyme in the process of oxidative decarboxylation of α-keto acids (for review, see ref *1*). This especially involves the transformation of pyruvate into acetyl-CoA. LA can be found in cells as either cyclic disulfide (LA) or in its reduced, open chain form as dihydrolipoic acid (DHLA). These two forms can quickly be converted into each other by redox reactions. LA is optically active, whereby the *R* form represents the naturally occurring enantiomer.

LA has successfully been employed in the treatment of diabetic polyneuropathy for decades (*2*). Its mechanism of action has been explained by its antioxidative potential (*1*), which was also thought to mediate the protective action of LA against mushroom poisoning or CCl_4-induced liver damage. An increasing number of data show not only that LA is cytoprotective due to its antioxidative properties but also that it activates specific cellular signalling pathways (*3*, *4*).

Hepatocyte apoptosis induced by ligands such as TNF-α contributes to the pathogenesis of a wide range of diseases, including viral hepatitis, alcoholic hepatitis, fulminant hepatic failure, ischemia/reperfusion injury (*5*), and sepsis (*6*). Inhibitors of hepatocyte damage might therefore be important therapeutics for the prevention/treatment of these diseases.

LA has been described to attenuate the production of TNF-α in Kupffer cells, the resident macrophages of the liver (*7*), and to protect from hepatic ischemia/reperfusion injury (*4*, *8*, *9*), which is known to be mediated to a major extent by TNF-α (*5*). Due to the interesting signalling properties and the safe applicability of LA, we aimed to determine whether LA can induce cytoprotective signalling pathways in hepatocytes. Since (*R*)-LA is the naturally occurring enantiomer, all experiments were performed with (*R*)-LA.

We used a common model of hepatocyte apoptosis: programmed cell death was induced by treatment of cells with the inflammatory cytokine TNF-α and the inhibitor of transcription Actinomycin D. According to Leist et al.,

† This work was supported by the DFG (FOR 440/1, project 2 and KI 702/2−2).

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[1] Abbreviations: A, ActD, actinomycin D; BHT, butylhydroxytoluol; DHLA, dihydro lipoic acid; HNMPA-$(AM)_3$, H, hydroxy-2-naphthalenylmethylphosphonic acid, trisacetoxymethyl ester; IR, insulin receptor; IRS-1, insulin receptor substrate 1, LA, (*R*)-α-lipoic acid; NAC, *N*-acetylcysteine; TNF-α, tumor necrosis factor α; WM, wortmannin.

10.1021/bi602547m CCC: $37.00
Published on Web 02/03/2007

TNF-α needs cotreatment with transcription inhibitors to induce apoptosis in isolated hepatocytes (*10*).

Comparing the action of LA to other antioxidants, our data revealed that LA mediates antiapoptotic action in hepatocytes in a unique way by the activation of the insulin signalling pathway.

EXPERIMENTAL PROCEDURES

Materials. LA was a gift from Viatris Inc. (Frankfurt/Main, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Biomol GmbH (Hamburg, Germany), the inhibitor of insulin receptor tyrosine kinase HNMPA-$(AM)_3$ was from Calbiochem (Bad Soden, Germany), and all other materials were either from Sigma (Deisenhofen, Germany) or VWR International (Munich, Germany).

Animals. Male Sprague−Dawley rats weighing 220−280 g were purchased from Charles River WIGA GmbH (Sulzfeld, Germany). The animals had free access to chow (Ssniff, Soest, Germany) and water up to the time of the experiments. All animals received humane care. The study was registered with the local animal welfare committee.

Isolation of Primary Hepatocytes. Hepatocytes were isolated using a modified two-step collagenase digestion method (*11*). Male Sprague−Dawley rats were anaesthetized by intraperitoneal injection of Pentobarbital (50 mg/kg body weight). Cell viability was determined via trypan blue staining. Hepatocytes were seeded into collagen R (0.2 mg/mL, Serva, Heidelberg, Germany)-coated 12- and 6-well culture plates (Peske, Aindling-Pichl, Germany) at a concentration of 8×10^5 and 10^6 cells/mL in L-15 medium (Pan Biotech, Aidenbach, Germany) containing 5% (v/v) FCS (Gibco Invitrogen Corporation, Karlsruhe, Germany) and insulin (125 U/L). After 4 h, cells were washed and made quiescent with insulin-free medium.

Caspase-3-Like Activity. Untreated or treated hepatocytes (8×10^5 cells/mL; 12-well tissue culture plates) were washed twice with ice-cold HBSS (0.95 mM $CaCl_2$, 5.3 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 136.75 mM NaCl, 0.34 mM Na_2HPO_4, 20 mM Hepes, pH 7.35), incubated with 50 μL of ice-cold lysis buffer (5 mM $MgCl_2$, 1 mM EGTA, 400 μL of Triton X-100, 25 mM Hepes, pH 7.5), and frozen at −85 °C overnight. After thawing on ice, caspase-3-like activity was determined according to the procedures of ref *12*. Generation of free fluorescent 7-amino-4-trifluoromethylcoumarin (AFC, Sigma, Deisenhofen, Germany) was measured kinetically after incubating at 37 °C (Fluostar, BMG GmbH, Offenburg, Germany). Control experiments revealed the linear character of activity regarding duration and protein concentration. Further control experiments demonstrated that LA does not interfere with the assay (*4*). Protein concentrations of the corresponding samples were quantified with the Pierce Assay (Pierce, Rockford, IL).

Lactate Dehydrogenase Efflux. Lactate dehydrogenase (LDH) release into the cell medium is a sensitive indicator of necrotic cell damage. The assay is based on the LDH-catalyzed conversion of pyruvate to lactate (*13*). Thereby, NADH is oxidized to NAD^+, resulting in a decrease in NADH extinction. For measurement, the following solutions were pipetted into a volume-reduced cuvette (Peske, Aindling-Pichl, Germany): 500 μL of phosphate buffer (50 mM K_2HPO_4 and 50 mM KH_2PO_4 are mixed until pH 7.5 is reached), 60 mM pyruvate, 500 μL of cell supernatant, and 500 μL of NADH solution (10 mg/mL NADH−Na_2 dissolved in 0.5% $NaHCO_3$ solution). The extinction was continuously monitored for 1 min at room temperature (RT) by a plotter. The enzymatic activity was calculated on the basis of the decrease in NADH extinction ($\epsilon_{365} = 3.34\ mM^{-1}\ cm^{-1}$).

Immunoprecipitation and Detection of Bad Phosphorylation. Untreated or treated hepatocytes grown in 6-well tissue culture plates (1×10^6 cells/mL) were harvested on ice by washing twice with ice-cold HBSS (see above), followed by addition of 100 μL of ice-cold lysis buffer (50 mM Hepes, 50 mM NaCl, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1% Triton X-100, pH 7.5) supplemented with Complete (Roche Diagnostics, Mannheim, Germany). After centrifugation, protein concentrations of the supernatant were determined by the Bradford Assay (BioRad Laboratories, Munich, Germany), and equal amounts of protein were adjusted by addition of lysis buffer. Total Bad was immunoprecipitated from the lysate after 12 h of incubation and gentle shaking with a phosphorylation state-independent anti-Bad antibody (1:200, Cell Signalling, New England Biolabs, Frankfurt/Main, Germany), followed by addition of 50 μL/500 μL of protein A agarose beads for 2 h at 4 °C. After centrifugation, the supernatant was discarded, and the beads were washed three times with ice-cold lysis buffer and resuspended in 50 μL 2.5× sample buffer. Immune complexes were cleared from the agarose beads by heating to 95 °C for 5 min and subsequent microcentrifugation. A 45 μL portion of the supernatant was used for SDS-PAGE and Western blotting.

Immunoprecipitation and Detection of Insulin Receptor Phosphorylation. A portion of hepatocytes (1×10^6/mL) was lysed in 100 μL of ice-cold lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% $C_{24}H_{39}O_4Na$, 1% Triton, 10% glycerol, 1 mM PMSF, pH 7.4) supplemented with Complete (Roche Diagnostics, Mannheim, Germany) and immediately frozen overnight. After thawing on ice, cells were incubated with an additional lysis buffer (140 mM NaCl, 10 mM KCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1% Triton, pH 7.5, supplemented with Complete) for 30 min on ice in a final volume of 900 μL. After centrifugation (14 000 rpm, 4 °C, 10 min), protein concentrations of the supernatant were determined by the Bradford Assay (BioRad Laboratories, Munich, Germany), and equal amounts of protein were adjusted by addition of lysis buffer.

The supernatants were precipitated by adding protein A agarose beads (previously incubated overnight at 4 °C with 1 μg of an anti-insulin receptor antibody from Upstate) for 2 h at 4 °C. The beads were collected by centrifugation (6000 rpm, 4 °C, 6 min), washed, and centrifuged (6000 rpm, 4 °C, 6 min) twice with buffer I (400 mM NaCl, 50 mM Tris/HCl, 0.1% Triton), followed by two additional washing and centrifugation (6000 rpm, 4 °C, 4 min) steps with buffer II (500 mM NaCl, 10 mM Tris/HCl). Subsequently, the proteins were solubilized in 60 μL 2× sample buffer, boiled for 3 min, and centrifuged for 1 min at 6000 rpm, and the

supernatants were immediately used for SDS−PAGE and Western blotting.

Western Blot. Western blots were performed essentially as described in ref *13* by dissolving the immunoprecipitated supernatant and employing an anti-phospho-Bad (Ser112 or Ser473) polyclonal antibody or an anti-phosphotyrosine antibody (Cell Signalling, New England Biolabs, Frankfurt/Main, Germany), followed by incubation with HRP-conjugated secondary antibodies. Finally, the immunoreactive bands were visualized with a chemiluminescent detection kit (ECL Plus, Amersham Pharmacia, Braunschweig, Germany) and subsequent exposure to a medical X-ray film (Fuji, Duesseldorf, Germany). To exclude loading differences, the blots were reprobed with antibody against the unphosphorylated form of Bad (Cell Signalling, New England Biolabs, Frankfurt/Main, Germany) or the insulin receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

PKA Activity. The phosphorylation activity of protein kinase (PKA) was measured in primary hepatocytes by in vitro phosphorylation of the highly specific peptide substrate kemptide with [γ^{32}P]-ATP (Amersham-Pharmacia, Braunschweig, Germany). The assay was performed with the PKA assay kit from Calbiochem (Schwalbach, Germany) according to the manufacturer's instructions (*14*).

Molecular Modeling. The crystal structure of the tyrosine kinase domain of the insulin receptor was retrieved from the Brookhaven Protein data bank (code 1IR3). GRID calculations were performed with version 22 of the GRID software (GRID, version 22; Molecular Discovery Ltd., 4 Chados Street, London, U.K., 2005). Three probes were used in the calculations: a methyl probe, a sulfur probe, and a carboxylate probe. On the basis of the results of GRID analysis, a LA molecule was manually docked into the postulated binding pocket. The stability of this binding site model was tested by means of molecular dynamics simulations using the program GROMACS (*15*). The protein structure was embedded in a cubic box containing 10 854 SPC (*16*) water molecules with periodic boundary conditions. The system was minimized using a steepest-descent algorithm and then equilibrated for 2 ns at a temperature of 310 K.

Insulin Receptor Kinase Activity. Insulin receptor was immunoprecipitated from 2×10^6 HepG2 cells (ATCC). Cells were grown in RPMI1640 containing 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Prior to precipitation, cells were cultivated for 8 h in serum-free medium. Cells were washed twice with ice-cold PBS and lysed in 500 μL of lysis buffer (50 mM Tris−HCl buffer, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF) supplemented with Complete (Roche Diagnostics, Mannheim, Germany) and immediately frozen overnight. After thawing on ice and centrifugation (14 000 rpm, 4 °C, 10 min), the clear supernatant was incubated with 2 μg of polycolnal anti-insulin receptor β (C-19) antibody (Santa Cruz Biotechnology) overnight at 4 °C. The immune complexes were collected by incubating the sample with protein A sepharose CL-4B (Amersham) overnight at 4 °C. The beads were collected as indicated above for precipitation of the IR. Exogenous phosphorylation activity was determined by a modification of the phosphocellulose adsorption method (*17*) using the IRS-1 peptide Y608 (Biomol, U.S.A.) according to ref *18*. Immunoprecipitated IR was incubated sequentially with 800 μM (*R*)-LA (30 min at RT) and 50 μM ATP/5 mM $MnCl_2$/50 mM HEPES (1 h at RT) for autophosphorylation. Substrate phosphorylation reaction was initiated by the addition of 400 μM IRS-1 peptide diluted in a mixture of 50 mM [γ-^{32}P]ATP/5 mM $MnCl_2$, 50 mM HEPES, and 0.1% Triton X-100. The reaction was allowed to proceed for 1 h. After 15 and 30 min, a 10-μL aliquot was taken and spotted directly on P81 phosphocellulose paper (Upstate). Paper squares were allowed to dry for 30 s and washed four times for 30 min each in 1 L of 75 mM phosphoric acid. The papers were dried, and incorporated phosphate was quantified using a phosphoimager (BAS-250, Fuji).

Statistical Analysis. All data are expressed as the mean ± SEM (n = number of independent cell preparations). Unless stated otherwise, all experiments were performed as triplicates with cells of at least three independent preparations. Statistical significance between groups was determined by Student's *t*-test. A p value of <0.05 was considered to be statistically significant. Statistical analyses were performed with GraphPad Prism (Version 3.02, GraphPad Software Inc., San Diego, CA).

RESULTS

LA Protects against TNF-α/ActD-Induced Hepatocyte Apoptosis. To test the effects of LA on apoptosis, primary hepatocytes were incubated for 2 h with LA, followed by 15 min of incubation with ActD (A) and then 12 h of incubation with TNF-α (T). As seen in Figure 1, panel A, pretreatment with LA significantly reduced T/A-induced caspase-3-like activity and, thus, apoptosis. We tested various stimulation sequences to determine when the addition of LA is most efficient to protect from apoptosis. We found the most pronounced protection after 2 h of pretreatment with LA (data not shown). Thus, we investigated the signal transduction pathway involved in protection against T/A-induced apoptosis with this stimulation pattern. We selected a concentration of 200 μM for all further experiments, since incubation of the cells with LA alone (in the absence of T/A) in concentrations higher than 400 μM slightly increased caspase-3-like activity (data not shown).

To rule out that cells become necrotic instead of apoptotic in the presence of LA we determined the LDH release in the presence or absence of LA. As expected, T/A induced secondary necrosis and, therefore, LDH release from hepatocytes. LA not only attenuated apoptosis but also was able to protect the cells against T/A-induced secondary necrosis (Figure 1, panel B).

Since LA's potency to protect from apoptosis has been suggested to be mediated due to its antioxidant action (*19*), we aimed to test whether other antioxidants were also able to protect hepatocytes in our model of T/A-induced apoptosis. In fact, some of the tested antioxidants, all employed in typical concentrations, induced protection from hepatocyte apoptosis (Figure 1, panel C). Whereas *N*-acetylcysteine (NAC) and vitamin E did not exhibit any protective properties, the water-soluble vitamin E derivative Trolox as well as butylhydroxytoluol (BHT) did protect hepatocytes from T/A-induced apoptosis (Figure 1, panel C). These data suggested that antioxidant properties of LA should at least partly contribute to its antiapoptotic effects.

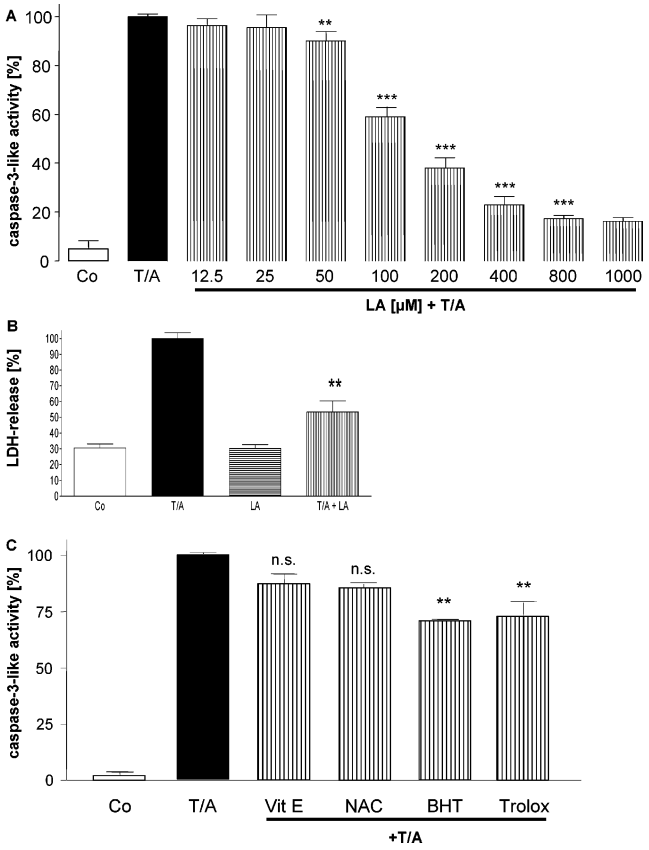

FIGURE 1: LA-preincubation significantly reduces TNF/ActD-induced apoptosis in primary rat hepatocytes. Hepatocytes either were left untreated (Co) or were treated for 2 h with various concentrations of LA or with different antioxidants (vitamin E/Vit E [60 μM], *N*-acetylcysteine [10 mM], butylhydroxytoluol [20 μM], or Trolox [2 mM]) prior to 15 min incubation with ActD (A, 0.2 μg/mL), followed by 12 h with TNF (T, 28 ng/mL) = T/A. A: Cells were lysed and caspase-3-like activity was measured (see Materials and Methods). Data are expressed as percent of enzyme activity of T/A-treated cells set as 100%. Columns show mean ± SEM of three independent experiments performed in triplicate. B: At the end of the incubation period, LDH-efflux was measured in the supernatant (see Materials and Methods). Data are expressed as percent of enzyme activity of T/A-treated cells set as 100%. Columns show mean ± SEM of two independent experiments. C: Cells were lysed after treatment, and caspase-3-like activity was measured (see Materials and Methods). Data are expressed as percent of enzyme activity of T/A-treated cells set as 100%. Columns show mean ± SEM of three independent experiments performed in triplicate. ***$p < 0.001$ and **$p < 0.01$ significantly different from T/A-treated cells.

Since the LA metabolite DHLA, its reduced form, is regarded as a more potent antioxidant than the oxidized mother compound, we aimed to determine the effects of DHLA on T/A-induced apoptosis. Interestingly, the compound rather strongly induced apoptotic cell death in hepatocytes (data not shown). These data suggested that the antioxidant action of LA is not solely responsible for its strong protective action, which seemed far more pronounced than that of other antioxidants. We therefore hypothesized a specific interaction with signal transduction pathways responsible for this antiapoptotic action.

LA Protects from Hepatocyte Apoptosis via Activation of the PI3-K/Akt Pathway. Since the PI3-K/Akt pathway is known to participate in antiapoptotic signalling cascades in different cells, among them hepatocytes (*20*), we investigated whether this pathway contributes to the antiapoptotic action

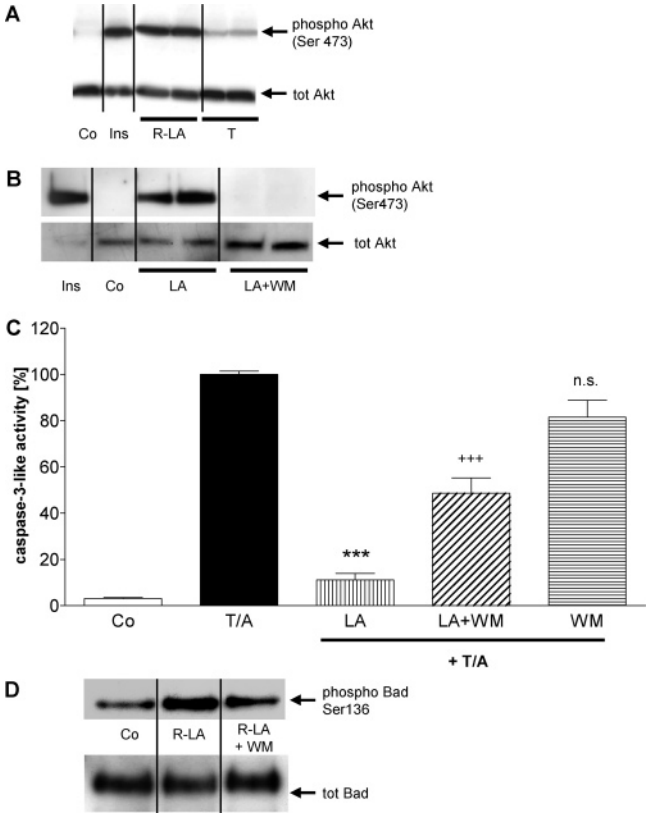

FIGURE 2: LA-mediated reduction of TNF/ActD-induced apoptosis is mediated by activation of the PI3-K/Akt pathway. A: Hepatocytes either were left untreated or were treated with LA [200 μM], insulin [50 ng/mL], or Trolox [2 mM] for 20 min. After incubation, Western blot analysis (see Materials and Methods) was performed to detect phosphorylated (phospho Akt Ser473) and total Akt (tot Akt) levels. The figure shows one representative blot out of three independent experiments. B: Hepatocytes were treated with or without insulin/Ins [50 ng/mL] or LA [200 μM] for 20 min and in some cases were preincubated for 30 min with wortmannin/WM [1 μM]. Equal amounts of DMSO, which was used to dissolve WM, were added to each well. The subsequent Western blot analysis (see Materials and Methods) was performed to detect phosphorylated and total Akt levels (phospho/tot Akt). The figure shows one representative blot out of three independent experiments. C: Hepatocytes were left untreated (Co) or were treated for 2 h with LA [200 μM] prior to 15 min of incubation with ActD (A, 0.2 μg/mL) followed by 12 h with TNF (T, 28 ng/mL) = T/A. To inhibit PI3-K, cells were preincubated for 30 min with wortmannin/WM [1 μM]. After incubation, cells were lysed and caspase-3-like activity was measured (see Materials and Methods). Data are expressed as percent of enzyme activity of T/A-treated cells set as 100%. Columns show means ± SEM of three independent experiments performed in triplicate. ***$p < 0.001$ significantly different from T/A-treated cells, $^{+++}p < 0.001$ significantly different from cells treated with T/A + LA; n.s., not significantly different from cells treated with T/A. D: Hepatocytes were either left untreated (Co) or were treated with LA [200 μM] for 8 h in the presence or absence of wortmannin/WM [1 μM, 30 min pretreatment] for different time points. After incubation, immunoprecipitation and Western blot analysis (see Materials and Methods) was performed to detect phosphorylated (phospho Bad Ser136) and total Bad (tot Bad) levels. The figure shows one representative blot out of three independent experiments.

of LA in hepatocytes. We could, in fact, show a strong activation of Akt upon treatment of hepatocytes with LA, which reached an intensity of that seen after treatment with insulin. In contrast, the water-soluble vitamin E derivate Trolox showed no significant activation of Akt (Figure 2, panel A). Inhibition of PI3-K by wortmannin (WM) com-

pletely abrogated the LA-induced activation of Akt (Figure 2, panel B), and incubation of hepatocytes with WM partly abrogated the LA-mediated protection against hepatocyte apoptosis (Figure 2, panel C).

Akt has been described to phosphorylate Bad, a proapoptotic member of the Bcl-2-family in vitro and in vivo and, thus, is able to inhibit Bad-induced apoptotic cell death in different cell types (*21*, *22*). Since LA incubation results in hepatocyte Akt phosphorylation, we investigated whether this kinase activation leads to a subsequent Bad phosphorylation at Ser136. Figure 2, panel D shows that LA phosphorylates and, thus, inactivates Bad downstream of PI3-K/Akt, since WM attenuated LA-induced Bad phosphorylation.

LA-Induced Activation of the Insulin Receptor Leads to Akt Phosphorylation. Activation of the insulin receptor (IR) is a prototypical event upstream of the PI3-K/Akt pathway. Since LA has been described as an insulin mimetic (*23*), we tested whether LA leads to an activation of the IR. Activation of IR is associated with tyrosine autophosphorylation of the intracellular domain of the receptor. Treatment of hepatocytes with LA led to a pronounced phosphorylation of the IR that was about as strong as that induced by insulin (Figure 3, panel A). In contrast, Trolox did not show activatory potential of the IR. Employment of the IR tyrosine kinase/autophosphorylation inhibitor HNMPA-$(AM)_3$ (hydroxy-2-naphthalenylmethylphosphonic acid, trisacetoxymethyl ester) (*24*) completely inhibited LA- and insulin-induced IR phosphorylation and Akt activation (Figure 3, panels B and C). These results showed that LA leads to Akt's activation via activation of the IR and PI3-K. Employing HNMPA together with LA to T/A-activated cells significantly attenuated LA's protective effect (Figure 3, panel D). Taken together, these data indicate that LA protects against T/A-induced hepatocyte apoptosis by activating the insulin receptor, leading to an activation of the PI3-K/Akt pathway.

No Bad Phosphorlyation via PKA. In addition to the Ser136 site, Bad can also be inactivated by phosphorylation of its Ser112 via PKA (*14*). Activation of the insulin receptor has previously been demonstrated to activate adenylyl cyclase, which can lead to activation of cAMP-dependent protein kinase (*25*). We therefore further investigated the possible participation of Bad phosphorylation by LA via PKA. The PKA activity assay, however, showed no significant difference in the kinase activation between untreated or LA-pretreated hepatocytes (Figure 4, panel A), whereas the membrane-permeable cAMP analogue Bt_2cAMP significantly increased PKA activity. We also could not demonstrate a PKA-dependent Bad phosphorylation on Ser112 either after LA or after insulin incubation (Figure 4, panel B). Thus, PKA is not involved in the LA-induced antiapoptotic signalling cascade, but activation of the IR/PI3-K/Akt pathway is crucial for LA's protective potential in hepatocytes.

Direct Binding of LA to the Insulin Receptor Tyrosine Kinase Domaine. Extracellular binding of insulin to its receptor leads to autophosphorylation of tyrosine residues in several regions of the intracellular β subunit of the receptor by activating the intrinsic tyrosine kinase activity. The activation of this IR tyrosine kinase activity is necessary for the interaction and subsequent activation of downstream signalling molecules. To test the mechanisms for how LA activates IR, we aimed to determine whether this activation is mediated by a direct binding of LA to the receptor. Computer modeling studies were performed according to ref

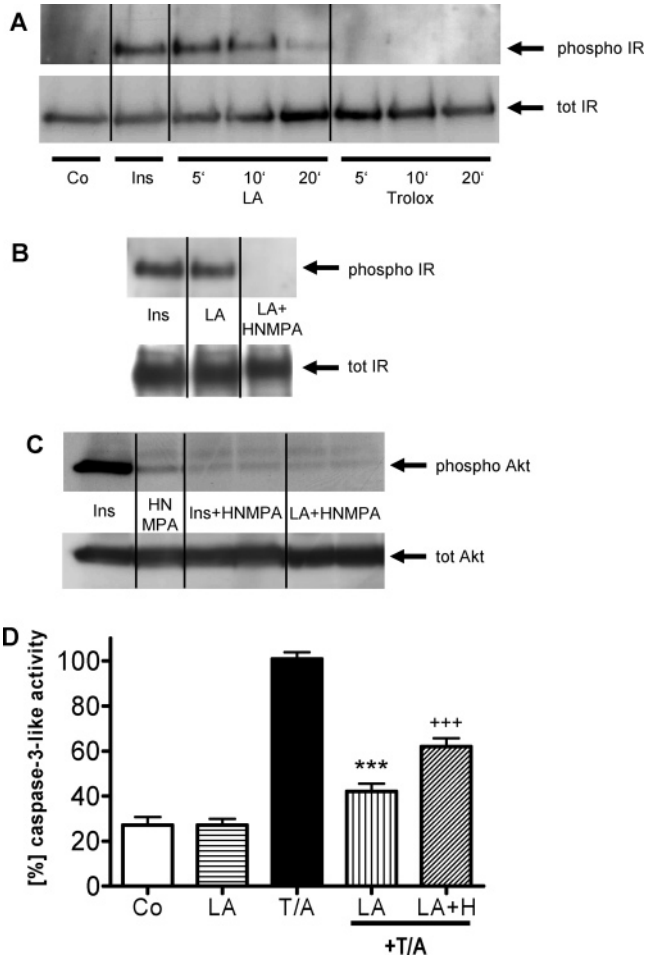

FIGURE 3: Insulin receptor activation mediates protection from T/A-induced apoptosis. A: Hepatocytes were treated with insulin/Ins [50 ng/mL] for 5 min or with LA [200 μM] or Trolox/T [2 mM] for different time points. After incubation, immunoprecipitation and detection of insulin receptor/IR phosphorylation was performed (see Materials and Methods). The figure shows one representative blot out of three independent experiments. B: Hepatocytes were treated for 5 min with insulin/Ins [50 ng/mL], LA [200 μM], or Trolox/T [2 mM] preincubated for 30 min $\pm$ HNMPA-$(AM)_3$ [200 μM]. The subsequent Western blot analysis (see Materials and Methods) was performed to detect phosphorylated and total insulin receptor levels after immunoprecipitation. The figure shows one representative blot out of three independent experiments. C: Hepatocytes were treated for 20 min with or without insulin/Ins [50 ng/mL], LA [200 μM], or Trolox/T [2 mM] and in some cases were preincubated for 30 min with the insulin receptor/IR inhibitor HNMPA-$(AM)_3$ [200 μM]. The subsequent Western blot analysis (see Materials and Methods) was performed to detect phosphorylated and total Akt levels (phospho/tot Akt). The figure shows one representative blot out of three independent experiments. D: Hepatocytes either were left untreated (Co) or were treated for 2 h with LA [200 μM], insulin/Ins [50 ng/mL] or Trolox/T [2 mM] prior to 15 min incubation with ActD (A, 0.2 μg/mL) followed by 12 h stimulation with TNF (T, 28 ng/mL) = T/A. For inhibition of IR phosphorylation, cells were preincubated for 30 min with HNMPA-$(AM)_3$/H [200 μM]. Cells were lysed and caspase-3-like activity was measured (see Materials and Methods). Data are expressed as percent of enzyme activity of T/A-treated cells set as 100%. Columns show mean $\pm$ SEM of three independent experiments performed in triplicate with $^{***}p < 0.001$ being significantly different from T/A-treated cells and $^{+++}p < 0.001$ being significantly different from T/A + LA-treated cells.

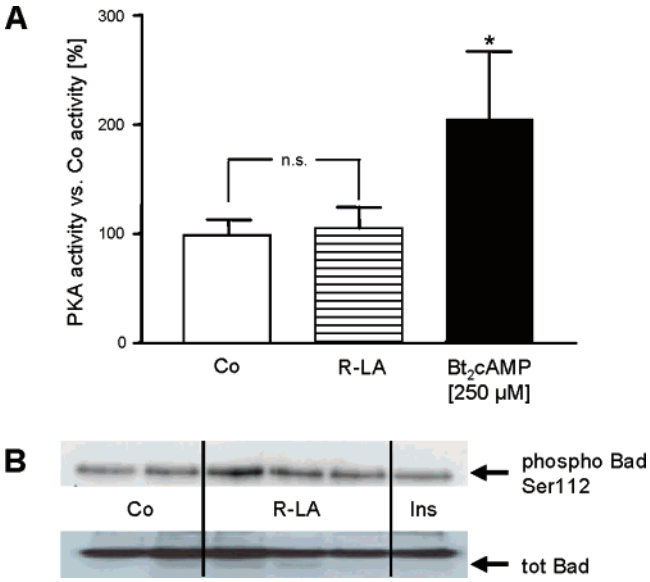

FIGURE 4: No PKA-dependent Bad phosphorylation. A: Hepatocytes either were left untreated (Co) or were treated for 2 h with LA [200 μM] or Bt_2cAMP [250 μM] as a positive control for 1 h. The PKA activity assay was performed as described under Materials and Methods. Specific activity is expressed as *x*-fold increase in comparison to control activity. Columns show means ± SEM of three independent experiments with $n = 3$ per group. $^*p < 0.05$ represents significant differences from untreated hepatocytes. B: Hepatocytes were treated with LA [200 μM] or insulin/Ins [50 ng/mL]. After incubation, immunoprecipitation and Western blot analysis (see Materials and Methods) was performed to detect phosphorylated (phospho Bad Ser112) or total Bad (tot Bad). The figure shows one representative blot out of three independent experiments.

26 using published structures of the tyrosine kinase domain of the insulin receptor (Figure 5, panel A, *27*−*29*).

The computer modeling studies, in fact, demonstrated that LA binds to the tyrosine kinase domain of the insulin receptor. To identify binding sites of LA, the program GRID was used. The program calculates interaction energies between specific functional groups and the protein and produces a map of possible binding pockets of the ligand. Figure 5, panel B shows the GRID fields in the IR tyrosine kinase domain for a sulfur, a methyl, and a carboxylate probe whereby the distance between the carboxylate field and the lipophilic sulfur and methyl field matches the distance between the acid group of LA and the dithiolan ring.

LA was then placed into the postulated binding pocket of the active kinase, and a molecular dynamics simulation was performed to investigate whether the three-dimensional structure of the protein remains intact in the presence of LA and whether LA, in fact, forms stable interactions in the binding pocket. Figure 5, panel C, shows the result of the molecular dynamics simulation: the three-dimensional structure of the protein remains intact, and LA has found a stable position in the binding pocket. Figure 5, panel D, shows a section of the binding pocket in detail: the carboxylate group of LA forms salt bridges with Lys 1168 and Arg 1131, whereas the dithiolane ring fills the lipophilic part of the binding pocket containing Leu1133, Pro1172, Met1176, Met1153, and Phe1186.

Most interestingly, our computer modeling data not only show that LA can bind to the tyrosine kinase domain of the IR, they also suggest that LA stabilizes the active form of the kinase: two amino acids of the LA binding pocket (Met1153 and Lys1168) belong to loop A of the tyrosine kinase domain of the IR. This loop A opens the binding pocket for ATP in the active form of the kinase. In contrast, it closes the ATP binding pocket in its inactive form.

Substrate Phosphorylation by LA-Treated IR. The computer modeling studies suggesting a direct interaction of LA with the IR were confirmed by an experimental approach. We immunoprecipitated the IR β-chain from untreated cells and treated the precipitate with (*R*)-LA. After a period of autophosphorylation, we added IRS-1, together with γ-^{32}P-ATP. IRS-1 was significantly more highly phosphorylated after 30 min of incubation, whereas at the 15-min time point, a clear tendency of higher phosphorylation could be observed. Unspecific kinase activity was very low, as indicated by a no-antibody control (Figure 6).

DISCUSSION

In this study, we obtained new insight into the molecular pharmacology of α-lipoic acid, a drug approved and successfully employed for the treatment of diabetic polyneuropathy. We demonstrated that LA protects hepatocytes from T/A-induced apoptosis by activating the insulin receptor pathway, followed by PI3-K/Akt signalling. Computer modeling studies suggest that this pathway is mediated by a novel process exerted by a small molecule: a direct binding of LA to the tyrosine kinase domain of the insulin receptor. These data are confirmed by the fact that LA induces substrate phosphorylation downstream of IR after immunoprecipitation.

Protection from Apoptosis by LA and Other Antioxidants. Our data demonstrate that LA has antiapoptotic potential in hepatocytes. Antiapoptotic action of LA has been previously described in the literature. LA inhibits retinal capillary cell death in diabetic rats in vivo (*30*) and protects primary neurons of the rat cerebral cortex against cell death induced by amyloid or hydrogen peroxide (*31*). In contrast, LA did not protect livers from postischemic apoptosis, whereas Akt activation did induce protection from postischemic necrosis (*4*). In addition, endothelial cells were not protected from oxidant-induced apoptosis by LA (*32*), but in contrast, LA even induced apoptosis when added to the cells alone. This effect was, as in our case, only observed in concentrations of several hundred micromolar up to 1 mM. LA concentrations as low as 100 μM potentiated Fas-mediated apoptosis of leukemic Jurkat cells, but not that of peripheral blood lymphocytes from healthy humans (*33*). These data suggest that, although LA has a certain potential to induce apoptosis in high concentrations, these concentrations might never be relevant in non-tumor cells in vivo. The safety of LA has, in fact, been demonstrated by its successful use as a drug without any severe side effects for decades (*2*).

Since LA is known to be partly metabolized into DHLA, the apoptosis-inducing effect of LA in concentrations higher than 400 μM might be exerted by DHLA formed inside the cells. In fact, DHLA rather potently induced cell death in our primary rat hepatocytes. In contrast to these data, rat thymocytes were protected by DHLA from methylprednisolone or etoposide-induced apoptosis, whereas LA was not efficient (*34*). It has to be noted, however, that the mechanisms of these effects have never been studied.

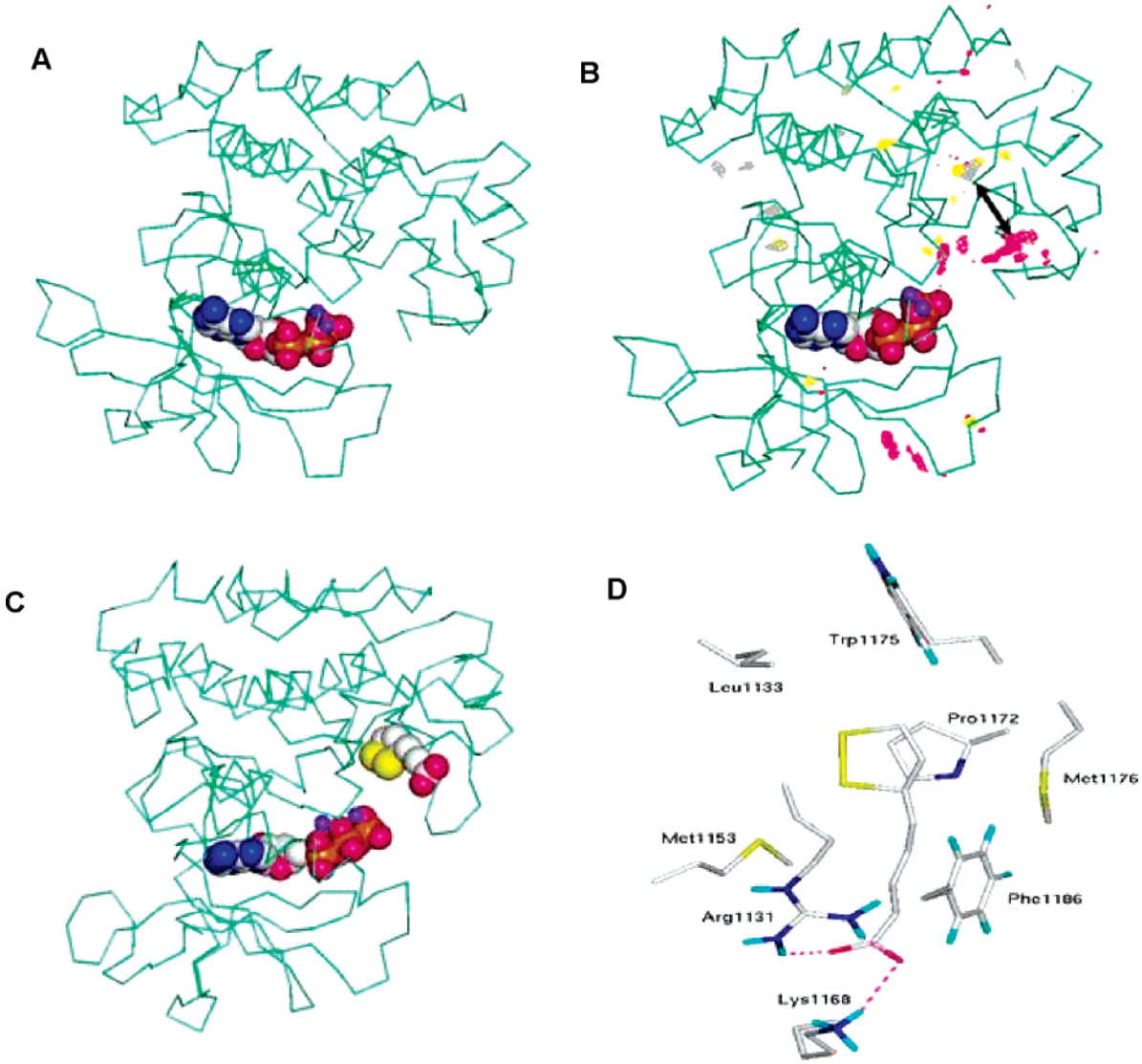

FIGURE 5: Computer modeling of binding of LA to the insulin receptor tyrosine kinase domain. A: Crystal structure (protein backbone in green) of the insulin receptor tyrosine kinase in its active form (with ATP). B: To identify binding sites of LA, the program GRID was used. The program calculates interaction energies between specific functional groups and the protein and produces a map of possible binding pockets of the ligand. The figure shows the GRID fields for a sulfur (yellow), a methyl (gray), and a carboxylate (red) probe. The distance between the carboxylate field (red) and the lipophilic sulfur and methyl (yellow and gray) fields matches the distance between the acid group of LA and the dithiolan ring (black arrow). C: LA was placed into the postulated binding pocket of the active kinase, and a molecular dynamics simulation was performed to investigate whether the three-dimensional structure of the protein remains intact in the presence of LA and whether LA in fact forms stable interactions in the binding pocket. The figure shows the result of the simulation: the three-dimensional structure of the protein remains intact, and LA has found a stable position in the binding pocket. D: A section of the binding pocket is shown in detail. The carboxylate group of LA forms salt bridges (purple) with Lys 1168 and Arg 1131. The dithiolane ring fills the lipophilic part of the binding pocket containing Leu 1133, Pro 1172, Met 1176, Met 1153, and Phe 1186.

In all of the cited papers, the protective action of LA was explained by its antioxidative action. Some authors even argue that a process is oxidant-mediated if it can be inhibited by LA (*19*).

We therefore tested whether antioxidant action is, in fact, involved in LA's cytoprotective effect. Employment of a set of antioxidants showed contradictory results: whereas BHT and Trolox significantly reduced T/A-induced apoptosis, vitamin E and NAC did not. Trolox has been previously described to attenuate T/A-induced hepatocyte apoptotic cell death (*35*), whereas to the best of our knowledge, none of the other antioxidants employed here has ever been published to affect T/A-induced apoptosis.

The small extent to which antioxidants attenuated hepatocyte apoptosis suggests that ROS only partly mediates T/A-induced cell death. Since LA in higher concentrations can almost completely abrogate apoptosis in our model, other signalling mechanisms have to add up with its antioxidative action (*19*).

Activation of the Insulin Receptor Protects from Apoptosis. The phosphorylation of IR followed by the activation of respective downstream targets induced by LA has previously been shown for smooth muscle cells and for adipocytes (*36*). It has been unknown, however, how LA might lead to the activation of the IR. Again, its redox-modulating potential had been hypothesized to contribute to this action (*23*).

Other antioxidants, among them Trolox, were even shown to abolish ROS-induced insulin receptor activation (*37*). Such observations are usually explained by the fact that oxidants

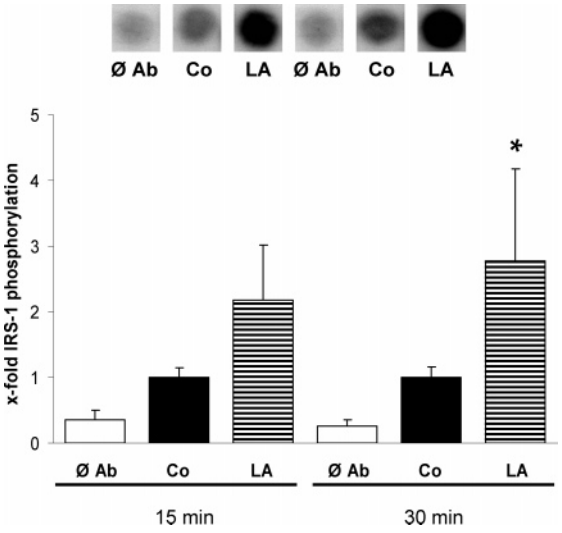

FIGURE 6: Kinase assay demonstrating LA action in immunoprecipitated IR. IR was precipitated from untreated HepG2 cells grown for 8 h under serum-free conditions with an anti-β-chain antibody. The precipitates either were left unstimulated (Co) or were treated for 30 min with LA [800 μM]. Control samples were treated like LA-stimulated samples, but IR-specific antibody was omitted (ø Ab). IRS-1 (Y608) phosphorylation was determined using the phophocellulose adsorption method. The phosphoimager images show one representative experiment at time points 15 and 30 min. The bar graph shows quantification data expressed as *x*-fold IRS-1 phosphorylation relative to nontreated control samples. Columns show means ± SEM of two independent experiments with $n = 2-3$ per group. *$p < 0.05$ represents significant differences from Co.

can inactivate protein tyrosine phosphatases, resulting in increased tyrosine phosphorylation of IR (*36*). In the past, a respective mechanism of action for LA has been ruled out by experiments measuring LA-induced IR tyrosine phosphorylation in the presence of tyrosine phosphatase inhibitors in the assay buffer (*36*).

Since the insulin receptor can also be directly modulated by alterations of the redox state of the cysteine residues present in IR α- and β-subunits, the authors hypothesized a potential effect of LA via alteration of the GSH status (*36*). Our data, shown *both* by computer modeling studies *as well as by a kinase activity assay in a cell-free system*, suggest that a major part of LA's activating action on the insulin receptor is mediated by a direct binding at the tyrosine kinase domain and possibly by a stabilizing function on loop A. Still, HNMPA(AM_3), although completely abrogating IR phosphorylation, only partially abrogated LA's antiapoptotic action. This observation suggests that the remaining effect of LA is, in fact, depending on its antioxidant action (see above).

Insulin receptor activation by LA has been demonstrated for the first time in adipocytes, and concentrations of LA of 2.5 mM, which is more than 10-fold our concentration, were employed (*38*). The cited work investigated LA's actions on glucose metabolism in adipocytes. Although the authors for the first time showed that LA is a non-insulin antidiabetic compound able to activate the insulin receptor, the group did not causally show that the action of LA on adipocyte glucose metabolism is, in fact, IR-dependent. By employing a specific inhibitor of IR tyrosine kinase phosphorylation, HNMPA(AM_3) (*24*), our data show for the first time that cytoprotective actions of LA are mediated *at least partially* via insulin receptor activation.

Insulin has been described to protect HepG2 cells and primary hepatocytes against apoptosis via activation of the PI3-K/Akt signalling cascade (*39*, *40*). Only the work performed in neonatal rat hepatocytes (*40*) further investigated potential downstream targets induced by insulin treatment. They observed an increased expression of antiapoptotic genes (Bcl-xL) and down-regulated proapoptotic genes (Bim and nuclear Foxo1). Our data provide evidence that IR-induced Akt activation leads to Ser136 phosphoryation of Bad that involves its inactivation and thereby leads to protection from apoptosis. Phosphorylation of Bad at Ser112, as induced by hepatocyte activation of PKA (*14*), however, could not be detected. Different from other insulin-treated tissue, such as muscle (*25*), activation of the IR did not induce activation of cAMP-dependent protein kinase in primary hepatocytes.

The remarkable decrease in mortality and prevention from sepsis in insulin-treated critically ill patients (*41*) might be connected with an IR-mediated protection from hepatocyte apoptosis: insulin has recently been shown to have antiapoptotic potential in hepatocytes of septic animals (*42*). Since hepatocyte apoptosis in sepsis is mediated via TNF-α (*6*) and LA has been shown to attenuate the production of proinflammatory factors contributing to the pathogenesis of sepsis in macrophages, Kupffer cells, and endothelial cells (*7*, *43*), these different actions might add up to an interesting pharmacological profile for the treatment of inflammatory diseases.

LA as a Directly Binding Activator of the Insulin Receptor. Our computer modeling studies demonstrate a direct binding site for LA to the tyrosine kinase domain of the IR. This is, to the best of our knowledge, the first respective bioinformatics approach to explain the pharmacological action of a small molecule activator of the insulin receptor by a potential direct interaction with the tyrosine kinase domain. The literature does report about a computer search approach to identify small molecules capable of binding the ligand binding domain of the insulin receptor by mimicking one epitope of the insulin molecule known to be involved in binding to the IR (*44*). The search revealed thymolphthalein as a candidate compound, which is an apparent weak agonist that displaces insulin from its receptor. The compound, in fact, led to a measurable phosphorylation/activation of the IR. However, it has to be noted that concentrations as high as 500 μM of the compound induced a markedly lower action than insulin (50 nM, equals ~290 ng/mL), whereas 200 μM LA in our assay led to roughly the same activation of IR as 50 ng/mL insulin. This suggests that binding to the tyrosine kinase domain is a more successful approach to mimick insulin action by a small molecule. In fact, an extracellular binding and mimicking of a binding epitope by a compound such as LA seemed rather unlikely, since LA is much smaller than not only insulin but also compounds such as the described thymolphthalein.

Our computer modeling studies suggesting a direct interaction of LA with IR were confirmed by an experimental approach in which LA treatment of immunoprecipitated IR caused substrate phosphorylation. Whereas Moini et al. previously showed a direct effect of LA on IR, inducing autophophorylation, our data, to the best of our

knowledge, show for the first time functional phosphorylation of an exogenous substrate upon binding of LA to purified IR (*36*).

In conclusion, LA as a directly binding activator of the insulin receptor together with its potent antioxidant potential might make it a promising drug for the prevention and treatment of diseases associated with hepatocyte apoptosis.

ACKNOWLEDGMENT

Stefanie Kulhanek-Heinze was supported by a scholarship from the University and Science Programme of the University of Munich. We thank Dr. Matthias Engel (Pharmaceutical and Medicinal Chemistry, Saarland University) for critically discussing the IRS-1 phosphorylation assay.

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BI602547M