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# Effects of Ethanol on Conformational Changes of Akt Studied by Chemical Cross-Linking, Mass Spectrometry, and $^{18}O$ Labeling

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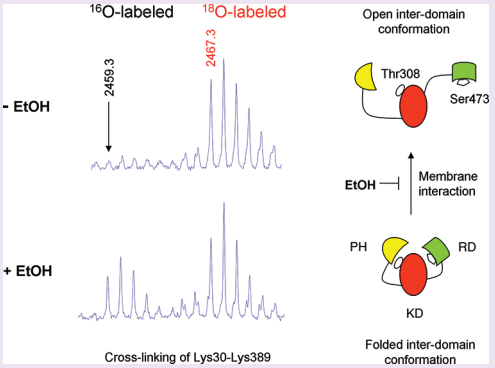
**ABSTRACT:** Although PI3K/Akt signaling that regulates neuronal survival has been implicated in the deleterious effects of ethanol on the central nervous system, underlying molecular mechanisms have not been fully elucidated. Akt−membrane interaction is a prerequisite step for Akt activation since it induces interdomain conformational changes to an open conformer that allows Akt phosphorylation by upstream kinases. In this study, we investigated the effect of ethanol on Akt activation by quantitatively probing Akt conformation using chemical cross-linking, $^{18}O$ labeling and mass spectrometry. We found that ethanol at pharmacologically relevant concentrations (20 or 170 mM) directly interacts with Akt and alters the local pleckstrin homology domain configuration near the $PIP_3$-binding site. We also found that ethanol significantly impairs subsequent membrane-induced interdomain conformational changes needed for Akt activation. The observed alteration of Akt conformation caused by ethanol during the activation sequence provides a new molecular basis for the effects of ethanol on Akt signaling. The *in vitro* conformation-based approach employed in this study should also be useful in probing the molecular mechanisms for the action of ethanol or drugs on other signaling proteins, particularly for those undergoing dramatic conformational change during activation processes such as members of AGC kinase super family.




The deleterious effects of ethanol on the various organs of the body have been well documented. In the central nervous system, excessive alcohol consumption has been shown to be associated with apoptotic cell death, learning and memory loss, and behavioral deficits.[1−3] One mechanism by which ethanol induces neurological disorders is by altering the growth factor-stimulated PI3K/Akt signaling pathway.[4−6]

Akt is a critical serine/threonine kinase in the PI3K pathway that controls neuronal cell survival.[7,8] The enzyme consists of three distinctive domain, including N-terminal pleckstrin homology (PH) domain (residues 1−120), C-terminal regulatory domain (residues 410−480), and a central kinase domain (KD).[9−11] Membrane interaction and phosphorylation are two subsequent steps involved in Akt activation. Cytosolic Akt is recruited to the plasma membrane by not only the well-established interaction of the PH domain with phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) generated by PI3-kinase upon growth factor stimulation[12−14] but also the recently discovered interaction of both PH and regulatory domains with phosphatidylserine (PS), which is the major anionic phospholipid class in eukaryotic biomembranes.[15] The Akt-$PIP_3$ and Akt-PS interactions result in interdomain conformational changes of Akt exposing Thr308 and Ser473 for phosphorylation by phosphoinositide-dependent protein kinases (PDKs).[16−18] Phosphorylation at both Thr308 and Ser473 activates Akt to phosphorylate and inhibit a number of downstream pro-apoptotic factors such as Bad, caspase-9, and forkhead transcription factors, thus promoting cell survival.[19−21]

Despite various attempts, the molecular mechanisms underlying the effects of ethanol on Akt signaling have not been fully understood. It has been reported that ethanol at 25−50 mM reduces the PI3-kinase activity and increases the level of PTEN (phosphatase and tensin homologue deleted on chromosome 10), which dephosphorylates $PIP_3$ to $PIP_2$, thereby attenuating Akt activity in cerebella.[4] We have previously found that ethanol exposure inhibits PS biosynthesis and lowers the PS level in developing rat brains.[22,23] The diminished Akt phosphorylation and increased apoptosis observed in neuronal cells[6] may be caused by inadequate PS-Akt interaction involved the Akt activation process.[15] In addition, it was found that in the liver ethanol intake promotes the binding of Akt to TRB3 (Tribbles homologue 3), thus preventing membrane association of Akt for subsequent activation.[5] A postulated mode of ethanol action was that ethanol substitutes the water surrounding biological macromolecules and membranes *via* competing for hydrogen bonding sites.[24,25] According to this postulation, ethanol can alter the conformation of proteins or disorder the phospholipid bilayer of membranes, prompting us to hypothesize that ethanol would interfere with Akt conformational changes that require the interaction with membranes.

We have previously used Akt−membrane interaction as a model system to probe the molecular basis of Akt activation by monitoring the interdomain conformational changes using chemical cross-linking, mass spectrometry, and $^{18}O$ labeling,[17]

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**Table 1. Detection of Cross-Linked Peptides by MALDI-TOF-TOF MS**

| | $[M + H]^+$ $m/z$ | | | | | |
|---|---|---|---|---|---|---|
| | 1611.0244 | 1793.0781 | 2459.2926 | 2940.4891 | 3121.4671 | 3465.7934 |
| cross-linked segments | Leu[155−168]Lys | Thr[371−386]Lys | Tyr[26−39]Lys Asp[387−391]Arg | Tyr[26−48]Arg | Glu[97−121]Arg | Leu[421−436]Arg Leu[277−289]Lys |
| domains involved | KD | KD | PH, KD | PH | PH | RD, KD |
| cross-linked lysines | Lys158-Lys163 | Lys377-Lys385 | Lys30-Lys389 | Lys30-Lys39 | Lys111-Lys112 | Lys426-Lys284 |
| error (ppm)[a] | 4 | 20 | 10 | 13 | 17 | 15 |

[a]Compared to the theoretical values.

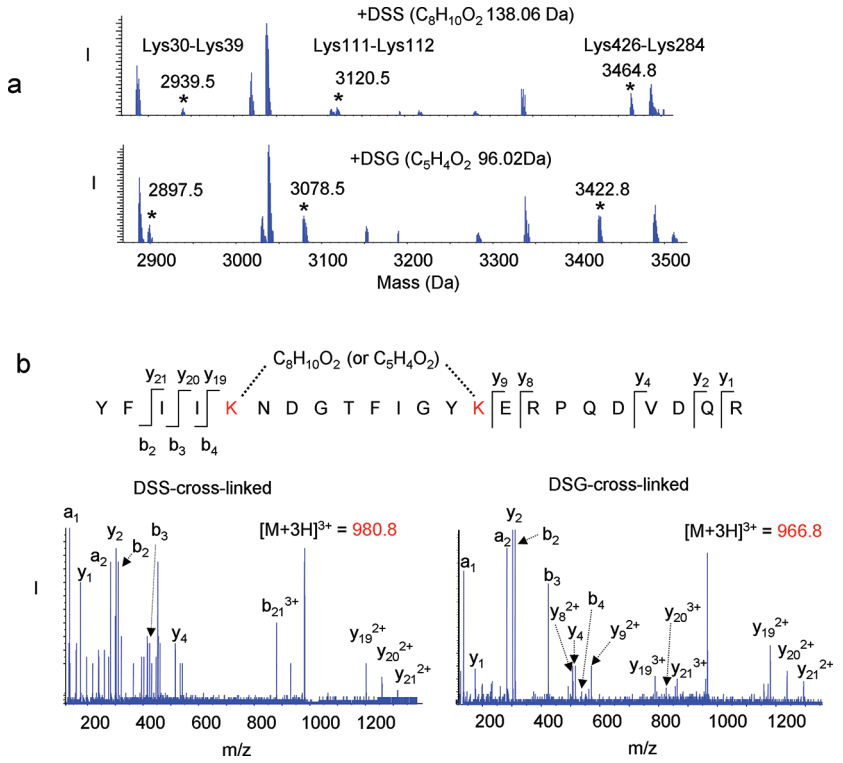


**Figure 1.** Representative nano-ESI-Qq-TOF mass spectra of tryptic digests from DSS or DSG modified Akt. (a) DSG-cross-linked peptides showing 42 Da lower mass compared to DSS-cross-linked peptides. Cross-linked peptides are marked with asterisks. (b) MS/MS spectra of the cross-linked peptides with mass of 2939.5 and 2897.5 Da, reconstructed from the triply charged ions at $m/z$ 980.8 and 966.8, respectively. The peptide sequence was assigned with single letter abbreviation. N-Terminal b ions and C-terminal y ions resulting from amide bond cleavage and N-terminal a ions due to the cleavage of C−C bond were labeled. The MS/MS data indicated that the cross-linked peptides were derived from Y[26−48]R (Tyr[26−48]Arg), with Lys30 linked to Lys39 *via* DSS or DSG.

as well as to investigate molecular mechanisms for various Akt inhibitors.[26] In the present study, the conformation-based approach allowed us to readily identify the effects of ethanol on Akt activation processes. We demonstrate that ethanol interacts directly with Akt and alters a local conformation of the PH domain near the $PIP_3$-binding site. Our findings further indicated that interdomain conformational changes after Akt−membrane interaction, which is required for Akt activation, were significantly impaired by ethanol, presumably due to the observed perturbation of the PH domain conformation and disruption of the membrane phospholipid bilayer.

## RESULTS AND DISCUSSION

**Ethanol Altered a Local Conformation in the PH Domain.** We first tested if ethanol interacts with Akt and alters its conformation at the resting condition. Chemical cross-linking combined with mass spectrometry was used to probe protein conformation as we previously reported.[17,26] Spatial distance information regarding the $\alpha$ carbons of lysine residues was obtained by identifying the lysine pairs cross-linked *via* DSG or DSS, an amine-reactive homobifunctional cross-linker with a 20 or 24 Å maximum cross-linking arm length, respectively. The cross-linking profile in the inactive Akt molecule with and without interaction with ethanol was quantitatively compared to probe the conformation status affected by ethanol.

From the tryptic digest of the DSS-modified inactive Akt molecule, we observed six intramolecular cross-linked lysine pairs including Lys111-Lys112 (3120.5 Da), Lys158-Lys163 (1610.0 Da), Lys377-Lys385 (1792.1 Da), Lys30-Lys39 (2939.5 Da), Lys30-Lys389 (2458.3 Da), and Lys426-Lys284 (3464.8 Da), using both MALDI-TOF-TOF (Table 1) and static off-line ESI-MS/MS (Figure 1). The results indicated that these lysine pairs are located within the 24-Å distance


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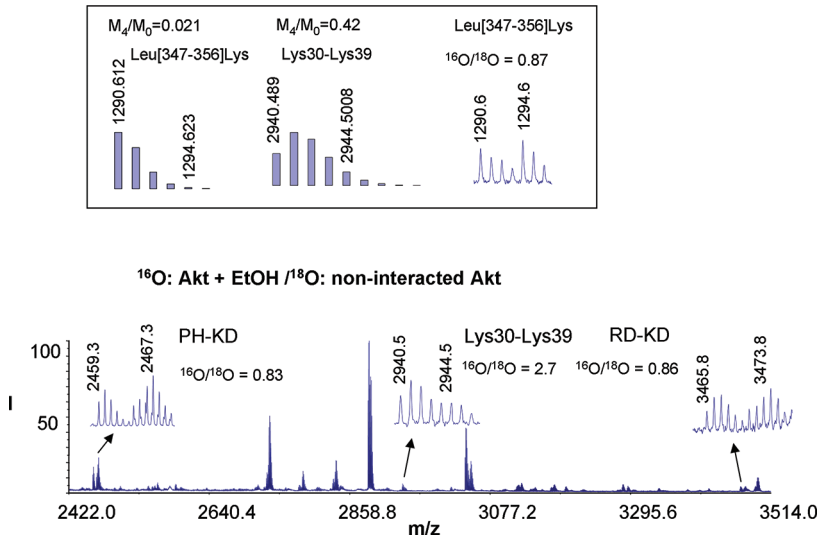


**Figure 2.** Representative MALDI-TOF-TOF MS spectrum of $^{16}O$-labeled tryptic digest from Akt interacted with ethanol (170 mM) mixed with $^{18}O$-labeled tryptic digest from non-interacted control. The spectrum of a representative peptide (Leu[347−356]Lys) used for normalizing the $^{16}O/^{18}O$ ratio is also shown in the inset. The average $^{16}O/^{18}O$ ratio for the non-cross-linked peptides was 0.91 ± 0.10. Inset also shows the theoretical isotopic distribution of the peptides with $m/z$ 1290.6 and 2940.5. $M_0$ and $M_4$ represent the theoretical relative intensities of the monoisotopic peak and isotopic peak at 4 Da higher mass, respectively. $M_4/M_0$ was used to correct the $^{18}O$-labeled peak intensities.

constraint. We also used DSG, a DSS analogue possessing a shorter cross-linking arm (maximum 20 Å), to refine the distance information for the lysine pairs.[17] These cross-linked peptides were identified by comparing the mass spectrum of the DSG- or DSS-modified sample with that obtained from non-cross-linked control. The DSG-modified Akt produced corresponding cross-linked peptide peaks at mass values that are 42 Da lower than those observed with DSS-cross-linking (Figure 1a). The identity of the peptides was revealed by MS/MS analysis. For example, Figure 1b shows the MS/MS spectra from the peptides with masses of 2939.5 (DSS-modified) and 2897.5 Da (DSG-modified) that were reconstructed from the triply charged ions at $m/z$ 980.8 and 966.8, respectively. These peptides were derived from peptide segment Tyr[26−48]Arg (Y[26−48]R) with the addition of $C_8H_{10}O_2$ or $C_5H_4O_2$ (the cross-linking arm of DSS and DSG, respectively) that bridged between Lys30 and Lys39 residues. Although the ESI-MS/MS often produced richer fragmentation information for sequence assignment, we also used MALDI-TOF-TOF MS for quantitation using $^{18}O$ labeling because of its superior capability of isotopic peak resolution and accurate mass measurement. The observed masses for all of the cross-linked peptides were within 20 ppm error compared with the theoretical mass values, further confirming the assignment of the cross-linking sites (Table 1).

To quantitatively compare the effect of ethanol on Akt conformation at the inactive stage, Akt was treated with ethanol and cross-linked with DSS, and the cross-linking profile was compared. Ethanol at a low pharmacologically relevant concentration (20 mM)[27−30] was selected for the experiments along with a high dose at 170 mM, which is in the blood ethanol concentration range shown to be reached in alcoholic patients with excessive drinking.[29] The tryptic peptides from DSS-modified Akt were labeled with $^{18}O$ water using immobilized trypsin as described in the experimental section, and those from the ethanol-treated DSS-modified Akt were similarly treated in the presence of $^{16}O$ water. In the presence of $H_2{}^{18}O$, trypsin catalyzes the exchange of two $^{16}O$ atoms for two $^{18}O$ atoms at the C-terminal carboxyl group of tryptic peptides, resulting in a mass shift of 4 Da in comparison to the peptides labeled in $H_2{}^{16}O$.[31,32] The differentially labeled samples were mixed and subjected to the analysis by MALDI-TOF-TOF MS as shown in Figure 2. Since $^{16}O$- and $^{18}O$-labeled peptides share the same chemical properties including ionization efficiency, an $^{18}O$-lebeled peptide serves as an ideal internal standard for accurately quantifying its corresponding counterpart based on the relative intensity of $^{16}O/^{18}O$ peptide peaks. The equation of $^{16}O/^{18}O$ ratio = $I_0/[I_4 - (M_4/M_0)I_0]$ was used for calculation, since complete labeling with two $^{18}O$ atoms (thus detected at 4 Da higher mass) was achieved for each tryptic peptide in this experiment.[26,32] $I_0$ and $I_4$ are the observed intensities for the peak appeared at the monoisotopic mass of the peptide without or with $^{18}O$ label, respectively; $M_0$ and $M_4$ are the theoretical intensities for the monoisotopic peak for the unlabeled peptide and isotopic peak with 4 Da higher mass, respectively. It is important to correct the intensity of $I_4$ ($^{18}O$ peak) using this equation, especially for larger peptides for which isotopic contribution of the nonlabeled peptide peak to $I_4$ becomes significant. For example, isotopic distribution of two unlabeled peptides with $m/z$ values of 1290.6 and 2940.5 are shown in the inset of Figure 2. The theoretical isotopic distribution predicts that 2% of the peak intensity of $I_4$ is contributed from $I_0$ for peptide Leu[347−356]Lys ($m/z$ 1290.6, $C_{59}H_{84}N_{15}O_{18}$), while 42% of $I_4$ is derived from $I_0$ for the cross-linked peptide of Lys30-Lys39 ($m/z$ 2940.5, $C_{135}H_{203}N_{34}O_{40}$). For through-space cross-linking involving two tryptic peptide segments, $^{18}O$-labeling resulted in an 8 Da increase in mass due to the incorporation of total four $^{18}O$-atoms into two C-termini. Two interdomain cross-linked peptides, Lys30 of Tyr[26−39]Lys in the PH domain linked to Lys389 of Asp[387−391]Arg in KD, and Lys426 of Leu[421−436]Arg in RD linked to Lys284 of Leu[277−289]Lys in KD, fell into this category (Figure 2). In such case, $^{16}O/^{18}O$ ratio was directly derived from $I_0/I_8$ because of negligible contribution of $I_0$ to $I_8$, where $I_8$ is the intensity of the fully labeled peptide peak detected at an 8 Da higher mass. The


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$^{16}O/^{18}O$ peak intensity ratio of five non-cross-linked tryptic peptides was used as an internal control for normalization. These peptides include Cys[77−86]Arg (1247.6 Da), Tyr[215−222]Arg (1052.5 Da), Val[145−154]Lys (1272.6 Da), Phe[407−419]Lys (1622.8 Da), and Leu[347−356]Lys (1289.6 Da). The average $^{16}O/^{18}O$ (+ethanol/−ethanol) peak intensity ratio for these peptides was 0.91 ± 0.10. As shown in Table 2 the normalized $^{16}O/^{18}O$ intensity ratio for the interdomain cross-linked peptides was close to 1, indicating that the interdomain conformation of Akt at the inactive stage was virtually unchanged by ethanol. However, the cross-linked peptide of Lys30-Lys39 in the PH domain was found to be substantially increased in the samples treated with ethanol at both 20 and 170 mM. According to the crystal structure of the PH domain (Protein Data Bank entry 1unp) the $\alpha$-carbon distance between Lys30 and Lys39 is 17.9 Å, which is well within the distance reached by the cross-linkers used in this study. Therefore, it is strongly suggested that accessibility to the Lys30/Lys39 pairs increased significantly by ethanol. Since no changes in the Lys30-Lys389 cross-linking were observed after ethanol treatment, it is likely that ethanol exposure caused Lys39 rather than Lys30 to be more accessible to cross-linkers, unless the increase in Lys30 exposure occurred simultaneously with decrease in Lys389 accessibility. In any event, it is clear from these data that interaction of Akt with ethanol altered a local conformation in the PH domain.

**Table 2. Effects of Ethanol on Akt Conformation**[a]

| | ethanol-interacted Akt/non-interacted Akt ($^{16}O/^{18}O$)[b,c] | | |
|---|---|---|---|
| ethanol (mM) | PH-KD (Lys30-Lys389) | intra PH (Lys30-Lys39) | RD-KD (Lys426-Lys284) |
| 20 | 1.03 ± 0.06 | 2.21 ± 0.40 | 0.96 ± 0.15 |
| 170 | 0.98 ± 0.09 | 2.25 ± 0.51 | 0.98 ± 0.04 |

[a]PH, pleckstrin homology domain; RD, regulatory domain; KD, kinase domain. [b]The $^{16}O$-labeled EtOH-treated sample was mixed with $^{18}O$-labeled Akt control for MS analysis. [c]Normalized to the average value of the non-modified peptides. The data represent the average of three independent experiments ± SD.

**Ethanol Altered the Interdomain Conformational Changes after Akt−Membrane Interaction.** Since ethanol altered the local conformation of Akt in the PH domain, and the binding of the PH domain to the plasma membrane is a critical step for the Akt activation process, we examined whether ethanol affects Akt−membrane interaction. We used the experimental strategy based on monitoring interdomain conformational changes of Akt induced by the membrane interaction as we established previously.[26] When Akt interacted with liposomes mimicking the inner leaflet of the plasma membrane under stimulated conditions ($PIP_3$/PS/PE/PC, 0.2:30:50:19.8), the interdomain cross-linking (Lys30-Lys389 or PH-KD, and Lys426-Lys284 or RD-KD) was no longer detected despite the fact that individual lysine residues were still accessible and modified by the cross-linkers. Upon Akt−membrane interaction the PH and RD domains moved away from the kinase domain farther than the spatial constraint of 24 Å allowed by the cross-linker DSS.[26] In the present study, we mixed $^{16}O$-labeled trypic digests from the membrane-interacted Akt with the $^{18}O$- labeled digest of the non-interacted Akt as a control and quantified the two interdomain cross-linked pairs as well as the intra-PH domain cross-linked peptide Lys30-Lys39.

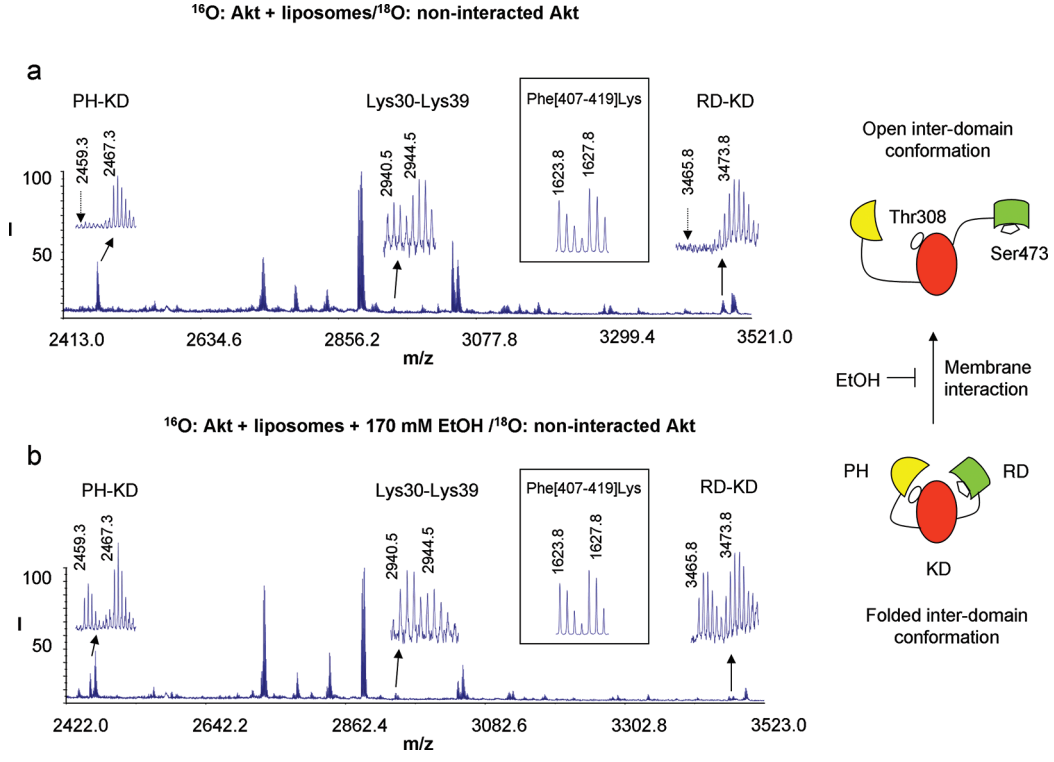


**Figure 3.** MALDI-TOF-TOF MS spectra obtained from $^{18}O$-labeled non-interacted control mixed with $^{16}O$-labeled membrane-interacted sample in the absence (a) or presence (b) of 170 mM ethanol. The spectrum of a representative peptide (Phe[407−419]Lys) used for normalizing the $^{16}O/^{18}O$ ratio is shown in the insets. The schematic presentation illustrates the inhibitory effect of ethanol on Akt interdomain conformational changes induced by membrane interaction.


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After membrane interaction, the interdomain cross-linked peptides observed with the non-interacted Akt ($m/z$ 2467.3 and 3473.8) were not detected ($m/z$ 2459.3 and 3465.8) ($^{16}O/^{18}O = 0$, Figure 3a and Table 3) as previously reported. [26]

**Table 3. Effects of Ethanol on Akt Intra- and Interdomain Conformational Changes Induced by Membrane Interaction**[a]

| | interacted Akt/non-interacted Akt ($^{16}O/^{18}O$)[b,c] | | |
|---|---|---|---|
| conditions for membrane interaction | PH-KD (Lys30-Lys389) | intra PH (Lys30-Lys39) | RD-KD (Lys426-Lys284) |
| Akt + liposomes | 0 | 1.08 ± 0.13 | 0 |
| Akt + 20 mM EtOH + liposomes | 0.45 ± 0.07 | 2.13 ± 0.37 | 0.32 ± 0.03 |
| Akt + 170 mM EtOH + liposomes | 0.65 ± 0.15* | 2.08 ± 0.35 | 0.54 ± 0.02** |

[a]PH, pleckstrin homology domain; RD, regulatory domain; KD, kinase domain. [b]The $^{16}O$-labeled sample was mixed with $^{18}O$-labeled non-interacted Akt control for MS analysis. [c]Normalized to the average value of the non-modified peptides. The data represent the average of three independent experiments ± SD. Statistical significance was tested against the case with 20 mM EtOH. *$p < 0.05$; **$p < 0.005$.

The intra-PH domain cross-linking did not change significantly after incubation with liposomes ($^{16}O/^{18}O = 1.08 \pm 0.13$, Table 3), suggesting that the membrane interaction did not cause the local conformational change in the PH domain, unlike the case with ethanol interaction.

To examine the effects of ethanol on membrane-induced Akt conformational changes, Akt was interacted with liposomes in the presence of ethanol (20 or 170 mM) and cross-linked with DSS. The $^{16}O$-labeled trypic digests from the membrane-interacted sample was mixed with the same $^{18}O$-labeled control digests used in Figure 3a, prior to the MS analysis. The interdomain cross-linked peptides that disappeared after membrane interaction (Figure 3a) were detected from the 170 mM ethanol-treated sample with intensity about 50−60% that of the $^{18}O$-labeled control (Figure 3, Table 3). This result indicated that around 50−60% Akt molecules were unable to undergo the interdomain conformational changes, apparently due to the disruption of Akt−membrane interaction by ethanol (Figure 3, schematic presentation). It is possible that perturbation of the PH domain caused by ethanol (Table 2) may have interfered with the interaction of Akt with membrane $PIP_3$ and PS. Alternatively, it is also possible that ethanol disrupted the membrane phospholipid bilayer, which in turn can impair Akt−membrane interaction, since ethanol at high concentrations (≥50 mM) has been shown to disorder the phospholipid acyl chain packing in some membranes.[25] To reduce the impact of ethanol on membrane integrity, we lowered the ethanol concentration to 20 mM and examined the conformational changes. The intra-PH domain Lys30-Lys39 cross-linking observed after membrane interaction similarly increased in the presence of ethanol at both 20 and 170 mM ($^{16}O/^{18}O = 2.13 \pm 0.37$ *vs* $2.08 \pm 0.35$ at 20 *vs* 170 mM ethanol, Table 3), indicating that ethanol at both concentrations caused comparable distortion of the local PH domain conformation. However, the $^{16}O/^{18}O$ ratios for the Lys30-Lys389 and Lys426-Lys284 interdomain cross-linked pairs ($0.45 \pm 0.07$ and $0.32 \pm 0.03$, respectively) indicated that membrane-induced interdomain conformational changes to an open conformer were affected less significantly at 20 mM ethanol in comparison to 170 mM (Table 3). These data suggest that ethanol at a high concentration (170 mM) may also perturb the membrane phospholipid bilayer, which can further impair Akt−membrane interaction.

**Discussion.** Akt activation has been considered a target for the ethanol action implicated in alcohol-related damages in numerous systems.[4−6] In this paper, we have presented a new molecular interaction mechanism by which ethanol affects Akt activation. Based on the biophysical and biochemical properties of ethanol to disrupt the hydrogen bonding in proteins and at the membrane−water interface, we hypothesized that ethanol may exert its action on Akt−membrane interaction, a critical step in Akt activation that enables conformational changes to allow the phosphorylation of Thr308 and Ser473 by PDK1 and mTORC2 respectively.[15,17]

The N-terminal PH domain of Akt plays a key role in Akt function by triggering the Akt membrane translocation through its binding to membrane $PIP_3$ produced upon growth factor stimulation.[9,11] According to the crystallographic data, Akt PH domain contains a $PIP_3$ binding pocket that is well-defined by a complex hydrogen bonding network through the ionic interactions between Lys14, Glu17, Arg25, Asn53, and Arg86, as well as several water molecules.[16,33] It has been established that Akt PH domain function depends on its conformation. Alteration in the conformation caused by disruption of the hydrogen-bonding network in the $PIP_3$ binding pocket has been shown to result in aberrant Akt activation. For example, substitution of Glu17 with lysine (Akt E17K) causes abnormal elevation of Akt localization to the membrane, leading to pathological activation of Akt,[33] whereas mutation of Arg25 to cysteine (Akt R25C) exerts an opposite effect.[34] It is well established that ethanol disrupts the hydrogen bonding in proteins and at the membrane−water interface.[24,25] By probing the three-dimensional structure of Akt in a physiologically relevant condition, we showed in the present study that direct interaction of ethanol with Akt induces a conformational change in the PH domain (Table 2). This local conformational alteration may have resulted from the disruption of the hydrogen-bonding network in the nearby $PIP_3$ binding pocket, through replacement of water molecules by ethanol.[24,25] In such case, it is expected that the Akt recruitment to membrane would be impaired. We tested this possibility by examining the effects of ethanol on interdomain conformational changes to an open conformer resulted from Akt−membrane interaction.[15,26] Indeed, we found significant reduction of the formation of an open conformer (Figure 3, Table 3), indicating that ethanol impairs Akt−membrane interaction, presumably due to altered PH domain conformation that in turn interferes with PH domain−$PIP_3$ interaction. It should be noted, however, that effects of ethanol on membrane binding can be caused by other mechanisms such as alteration of different Akt sites, which could not be probed by the cross-linking approach used in this study.

Well-established synergistic phosphorylation of Thr308 and Ser473[9] suggests that the interdomain conformational changes exposing Thr308 and Ser473 also occur synergistically upon membrane interaction. Akt−membrane interaction triggered by the tight binding between the PH domain and $PIP_3$ is thought to be strengthened by the electrostatic interaction of both PH and RD domains with membrane PS,[15] although it is not entirely clear at present whether these interactions occur concurrently or sequentially. It is noticed in this study that the PH-KD cross-linking is more significantly affected by ethanol


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than the KD-RD cross-linking (Table 3). The apparent differential effects of ethanol on membrane-induced configuration changes suggest that the membrane interaction of the PH domain is more susceptible to ethanol than the RD domain. Unlike the PH domain, the membrane binding of the RD is primarily derived from the interaction with PS.[15] It is plausible that the strong electrostatic binding between RD and PS established concurrently with or subsequent to the $PIP_3$-triggered initial membrane recruitment of Akt may be more resilient to the ethanol interruption.

We have previously demonstrated that membrane-induced Akt conformation changes regulate Akt phosphorylation of Thr308 and Ser473 by PDK1 and mTOR/rictor, respectively.[15] To validate if the ethanol-induced impairment of conformational changes leads to impaired phosphoylation by PDK1 and mTOR/rictor, effects of ethanol on these upstream enzymes first need to be examined. Like Akt, PDK1 activation depends on the interaction with membrane $PIP_3$ and PS through its PH domain.[18,35] In addition, insulin-induced phosphorylation of PDK1 has been shown to be reduced in the rat liver after prenatal ethanol exposure.[36] Interestingly, a recent report also suggests an association of increased mTOR/rictor activity with ethanol exposure.[37] These findings suggest that distinguishing Akt phosphorylation that is specifically linked to ethanol-derived Akt conformational changes is an extremely difficult task in either *in vivo* or *in vitro* systems.

We also found that ethanol impairs the membrane-induced interdomain conformational change in a dose-dependent manner. Although perturbation of the PH domain was similar at the pharmacologically relevant doses tested in the study (Tables 2 and 3), conformational changes were further impaired at a high dose (170 mM) in comparison to a low dose (20 mM) ethanol (Table 3). It has been demonstrated that ethanol at a high concentration impairs the membrane integrity.[25] It has been shown by crystallographic data that, away from the $PIP_3$ binding pocket, the Akt PH domain contains a highly positively charged surface consisting of Arg15, Lys20, Arg67, and Arg69.[11] We have recently demonstrated that these residues interact with negatively charged membrane phosphatidylserine (PS) through hydrogen bonding.[15] Since Akt activation relies on not only the specific binding to $PIP_3$ but also electrostatic interaction with PS,[15] it is conceivable that the disruption of the hydrogen bonding due to the loss of membrane integrity may have significant impact particularly on the Akt binding to PS. In a recent study, it has been reported that ethanol decreases cell proliferation and viability of human hepaRG cells in a dose (10−200 mM)-dependent manner, and ethanol-induced dysfunction in iron metabolism was proposed as one of the underlying mechanisms.[29] Because Akt is a key enzyme that controls cell proliferation and survival, our findings on the action of ethanol exerted on Akt−membrane interaction provides another molecular explanation for the observed antiproliferative effect of ethanol.

Proposed mechanisms for the impact of ethanol on Akt activation include inhibition of $PIP_3$ production[4] and PS biosynthesis[6] and increased expression of TRB3 that interferes with Akt membrane translocation through competitive binding to Akt,[5] as depicted in Figure 4. The results from the present study led us to propose another mechanism for the action of ethanol on Akt signaling. We demonstrated that ethanol disrupts interdomain conformational changes required for exposing Thr308 and Ser473 for subsequent phosphorylation and activation (Figure 4). This new mechanism enhances the current understanding of the biological effects of ethanol on Akt activation. Even if ethanol-induced abnormal production/degradation of signaling phospholipids or TRB3 expression could be corrected, Akt signaling might still be impaired due to direct effects of ethanol on Akt conformation. The *in vitro* conformation-based approach using chemical cross-linking and quantitative mass spectrometry used in the study should also be useful in probing the molecular mechanisms for protein−drug and protein−membrane interactions, particularly for proteins that undergo dramatic conformational change during activation processes, such as members of the AGC kinase super family.[38,39]

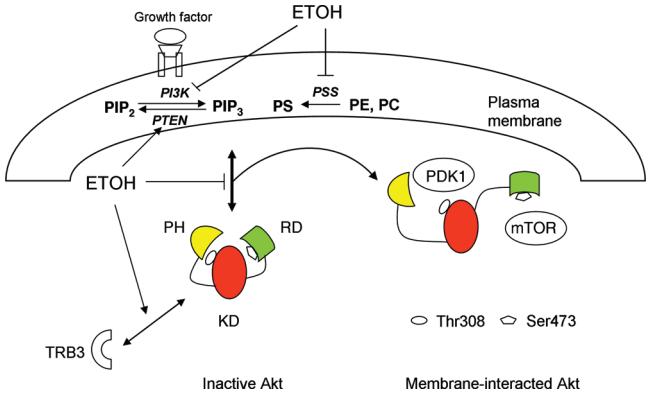


**Figure 4.** Proposed mechanisms for the inhibitory actions of ethanol on Akt activation. Ethanol has been shown to impair Akt signaling by decreasing the PI3 kinase activity and increasing PTEN expression, inhibiting PS biosynthesis, and promoting the Akt binding to TRB3, a negative regulator of Akt activation. An additional mechanism is proposed from the present study that ethanol disrupts Akt−membrane interaction, impairing the interdomain conformational change of Akt to expose Thr308 and Ser473 for subsequent phosphorylation and activation. mTOR, mammalian target of rapamycin; PC, phosphatidylcholine; PDK, phosphoinositide-dependent protein kinase; PE, phosphatidylethanolamine; PH, pleckstrin homology; PI3 kinase, phosphoinositide-3 kinase; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; $PIP_3$, phosphatidylinositol 3,4,5-trisphosphate; PS, phosphatidylserine; PSS, phosphatidylserine synthase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RD, regulatory domain; TRB3, Tribbles homologue 3.

## METHODS

**Materials.** Ethyl alcohol (200 proof) was purchased from The Warner-Graham Company. Inactive Akt1 was purchased from Upstate Cell Signaling Solutions. Disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG) were purchased from Pierce. Sequencing grade modified trypsin was purchased from Promega. Immobilized trypsin was obtained from Applied Biosystems. 1-Stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phospho-L-serine (18:0, 22:6-PS), 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (18:0, 22:6-PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0, 18:1-PC), and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol-3,4,5-trisphosphate (18:0, 20:4-$PIP_3$) were purchased from Avanti Polar Lipids. Pure water was obtained from a Gemini high purity water system. $H_2{}^{18}O$ (97%), cyclohexane, 2,6-di-*tert*-butyl-*p*-cresol (BHT), and diethylenetriamine pentaacetic acid (DTPA) were purchased from Sigma. $\alpha$-Cyano-4-hydroxycinnamic acid was purchased from Agilent Technologies. Other reagents were purchased from Sigma or Quality Biological, Inc.

**Unilamellar Vesicles Preparation.** Unilamellar vesicles (1 mg $mL^{-1}$) mimicking the lipid composition in the inner leaflet of neuronal plasma membrane (PC/PE/PS/$PIP_3$ 19.8:50:30:0.2) were prepared according to the method reported previously.[17] Briefly, PE (18:0,


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22:6), PC (16:0, 18:1), PS (18:0, 22:6), and $PIP_3$ (18:0, 20:4) at 1 mg $mL^{-1}$ each were mixed at the desired proportion. The mixture was dried under an $N_2$ steam, redissolved in 2 mL of cyclohexane containing 75 $\mu$M BHT, and lyophilized for 2 h under vacuum. The sample was reconstituted in 1 mL of PBS (pH 7.4) containing 50 $\mu$M DTPA. The lipid suspension was extruded 10 times through a 0.1 $\mu$m polycarbonate membrane (Corning, Inc.) using an Avanti mini-extruder (Avanti Polar Lipids). All of the above procedures were carried out in an argon box except the drying and lyophilizing steps. An aliquot of the sample was analyzed by high performance liquid chromatography–mass spectrometry (HPLC–MS) to verify the final concentrations of lipid components.[23]

**Chemical Cross-Linking Reaction.** Akt sample at 2.5 $\mu$M was dialyzed overnight against 50 mM HEPES (pH 7.4) containing 50 mM NaCl at 4 °C to remove the primary amine-containing Tris-HCl buffer. Five microliters of Akt was incubated with 30 $\mu$L of liposomes in the presence or absence of EtOH at 30 °C for 40 min. Alternatively, Akt was incubated at 30 °C for 40 min with or without EtOH in PBS buffer. In this study we used 20 and 170 mM ethanol to represent low and high pharmacologically relevant doses, respectively.[27−30] The mixture was incubated with a 50-molar excess of freshly prepared DSS in DMSO (made to a final concentration of DMSO of 1%) at RT for 10 min. At this cross-linking condition intermolecular cross-linked dimers or multimers were not observed according to SDS-PAGE analysis. The cross-linking reaction was quenched by adding 1 M Tris-HCl (pH 7.4) to a final concentration of 50 mM.[31]

**Tryptic Digestion and $^{18}O$ Labeling.** The cross-linked sample was digested with sequencing grade modified trypsin at 37 °C overnight using a trypsin to protein ratio of 1:20. After desalting using C18 Ziptip (Millipore Corp.), the sample was lyophilized to dryness. $^{16}O/^{18}O$ labeling was carried out similar to the method previously reported.[26] The dried peptides were reconstituted with 20 $\mu$L of acetonitrile and 100 $\mu$L of 50 mM $NH_4HCO_3$ in either regular $H_2{}^{16}O$ water or 97% $H_2{}^{18}O$. One-half microliter of 2 M $CaCl_2$ and 5 $\mu$L of immobilized trypsin were added to the digests. The mixtures were continuously rotated in an incubator at 30 °C for 30 h. After the samples were centrifuged at 15,000*g* for 5 min, supernatant was collected and acidified to pH 3.5 using TFA solution. The samples were concentrated using a SpeedVac (Thermo Savant). The DSS-modified Akt control sample (Akt + PBS) was labeled with 97% $H_2{}^{18}O$, while other samples prepared in different conditions were labeled with $H_2{}^{16}O$. The $^{18}O$-labeled sample was mixed with each of $^{16}O$-labeled samples and desalted with C18 Ziptip prior to mass spectrometric analysis.

**MALDI-TOF-TOF MS Analysis.** The sample containing $^{16}O$- and $^{18}O$-labeled peptides was mixed with matrix solution ($\alpha$-cyano-4-hydroxycinnamic acid) with a ratio of approximate 1:1. One microliter of the mixture was spotted on a MALDI plate. Samples were allowed to air-dry and analyzed by a 4700 MALDI-TOF-TOF Proteomics Analyzer (Applied Biosystems) operated in reflector positive ion mode. The UV laser (Nd:YAG) was operated at 200 Hz with wavelength of 355 nm. For MS analysis, a 1000–4000 $m/z$ mass range was used, typically with 5000 shots per spectrum. A 1-keV collision energy was used in MS/MS acquisition for precursor ions of interest. All acquired spectra were processed using 4000 Series Explore software (Applied Biosystems) in a default mode.

**Nanoelectrospray Ionization (ESI) Mass Spectrometric Analysis.** Desalted peptides were analyzed by a high resolution QSTAR pulsar Qq-TOF (quadrupole-time-of-flight) mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a nanoelectrospray ionization source. The ion source voltage was set to 1100 V in the positive ion mode. A full mass spectrum was acquired over an $m/z$ range of 500–2000. Ions of interest were subjected to collision-induced dissociation (CID) using high purity nitrogen to obtain MS/MS data. Resolution greater than 8000 and mass accuracy with less than 50 ppm error were attained in both full MS and MS/MS modes. The reconstructed mass spectral data were generated using Analyst QS 1.1 software (Applied Biosystems/MDS Sciex).

**Analysis of the Cross-Linked Peptides.** The cross-linked peptides were identified by comparing the mass spectrum obtained from DSS-modified sample with non-modified control. MS/MS analysis was used to determine the cross-linked lysine residues. Protein Analysis Work Sheet (PAWS) was used to assign the mass values of tryptic peptides. MS/MS data for the cross-linked peptides were manually interpreted with the assistance of PAWS, Analyst QS 1.1 software, and $^{18}O$ labeling. The theoretical relative intensities for the monoisotopic peak and the peak with 4 Da higher mass were obtained using Analyst QS 1.1 software.

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## ABBREVIATIONS

DSG: disuccinimidyl glutarate; DSS: disuccinimidyl suberate; ESI: electrospray ionization; KD: kinase domain; MALDI-TOF-TOF: matrix assisted laser desorption/ionization-time-of-flight-time-of-flight; MS: mass spectrometry; MS/MS: tandem mass spectrometry; PDK: phosphoinositide-dependent protein kinase; PH: pleckstrin homology; PI3 kinase: phosphoinositide-3 kinase; $PIP_2$: phosphatidylinositol 4,5-bisphosphate; $PIP_3$: phosphatidylinositol 3,4,5-trisphosphate; PS: phosphatidylserine; PSS: phosphatidylserine synthase; PTEN: phosphatase and tensin homologue deleted on chromosome 10; RD: regulatory domain; TRB3: Tribbles homologue 3

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