# Herbicide acetochlor inhibits adrenergic receptor-mediated calcium oscillations in rat hepatocytes

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**Abstract** Herbicide acetochlor after application enters both surface and underground waters, so that acetochlor concentrations in rivers and streams could be sufficiently high (nmol · $L^{-1}$) to exert long-term effects in aquatic animals. Occupational exposure may pose greater risks due to skin and inhalation exposures, resulting in much higher transient blood acetochlor concentrations (μmol · $L^{-1}$). Previous works have identified liver as the most susceptible toxicological target for acetochlor, and cytosolic calcium is known to play a vital role in multiple hepatocyte functions. Therefore the present work aims to examine acetochlor effect on calcium oscillations induced by adrenergic receptor activation in freshly isolated rat hepatocytes. It was found that acetochlor at lower concentrations (1, 10 μmol · $L^{-1}$) added simultaneously with phenylepinephrine (PE) had no effect on PE-induced calcium oscillations, but inhibited reversibly calcium oscillations at higher doses (50, 100, 200 μmol · $L^{-1}$) of acetochlor in selected hepatocytes. Brief prior acetochlor exposure (1, 10, 100 μmol · $L^{-1}$), however, had no apparent effect on either basal calcium or subsequent PE-induced calcium oscillations. Immunocytochemistry identified non-uniform α1 adrenergic receptor expression among isolated hepatocytes. Immunohistochemistry of liver slices revealed a distinct pattern of α1 adrenergic receptor density in liver lobules: a gradual low-to-high receptor density gradient from pericentral to periportal regions. This receptor density gradient is likely related to acetochlor inhibition in selected hepatocytes. Taken together it is concluded that acetochlor at blood concentrations attainable during acute skin exposure or acetochlor poisoning may exert transient inhibitory effects on α1 receptor signaling in the liver.

**Keywords** acetochlor; adrenergic receptor; calcium oscillations; hepatocytes

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## 0 Introduction

Pesticides are widely used to prevent loss in agricultural production. A sizable portion of agricultural pesticides used are herbicides, including chloroacetanilide herbicides such as acetochlor[1]. Annual acetochlor use both in industrialized US and in developing China is in the tens of millions of pounds[2−4].

Acetochlor has high water solubility, low soil adsorption, therefore is easily transported from farm fields by surface runoff[5−6]. Acetochlor concentrations in surface waters have been found to be in the nanomolar range: 0.926 nmol · $L^{-1}$[7], 0.63 nmol · $L^{-1}$[8], 1.11 nmol · $L^{-1}$[9], 2.85 nmol · $L^{-1}$[10], 9.3 nmol · $L^{-1}$[11], 0.19~2.67 n mol · $L^{-1}$[12], 6.15 nmol · $L^{-1}$[13], 3.91 nmol · $L^{-1}$ (1054.90 ng · $L^{-1}$)[14]. Atmospheric acetochlor has also been detected, values of up to 53.5 ng · $m^{-3}$ (198.15 nmol · $L^{-1}$) has been found[15]. Acetochlor concentration in the rainwater therefore could be up to 9.27 nmol · $L^{-1}$[16], 16.26 nmol · $L^{-1}$[17]. Due to its high soil mobility, the presence of acetochlor in soil has been found to be rather transient[18−20]. Acetochlor soil runoff into surface water and infiltration to underground water, aerosolized acetochlor precipitated in rain water, together form critical routes of

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acetochlor dispersion before moving into the ocean[18, 21–22].

Compared with acetochlor in the environment, occupational exposure to acetochlor poses greater harm to human health. Acetachlor metabolites are readily detected in the urine of farmers[23] and farm children[24], with seasonal variations[23, 25]. Urine acetochlor up to 0.26 nmol · $L^{-1}$ has been detected in pregnant women residing close to fields of acetochlor application[26]. Prenatal exposure to acetochlor and other herbicides has been correlated to reduced birth weight in human infants[27–28].

The effect of long-term and chronic exposure to nanomolar acetochlor has been extensively investigated. It has been found that exposure to environmental concentrations of acetochlor increased thyroid hormone receptor expression in aquatic animals[29–31]. Acetochlor-spiked feed increased estrogen receptor density in the rat[32–33]. Acetochlor accelerated frog tadpole metamorphosis via increased expression of the thyroid hormone receptor[34–35].

Long-term low level acetochlor exposure notwithstanding, effect of short-term occupational exposure at higher concentration has been less well studied. Micromolar acetochlor concentrations have been reported in the serum of acutely poisoned patients (18.61 μmol · $L^{-1}$)[36], in skin-exposed monkeys (0.37 μmol · $L^{-1}$)[37]. Further, human skin exposure (at 9.27 mmol · $L^{-1}$) could lead to contact dermatitis[38]. Therefore the present work aims to examine acetochlor effect on calcium signal in the freshly isolated rat hepatocytes. Since the α1 adrenergic receptor plays a prominent role in calcium signaling in hepatocytes[39–43], acetochlor effect on adrenergic receptor-mediated calcium oscillations was examined. It was found that micromolar acetochlor showed reversible inhibition of PE-induced cytoslic calcium oscillations in hepatocytes. Due to the universal role of calcium signaling in multiple cell types, this new finding has potentially important bearings on the occupational health problems of acetochlor.

# 1 Materials and methods

**1.1 Materials** Acetochlor [2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide, MWt 269.77], phenylepinephrine (PE), nuclear stain Hoechst 33342 were from Sigma-Aldrich (St. Louis, MO, USA). Collagenase H, 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) were from Calbiochem (Darmstadt, Germany). Amino acid mixture (MEM×50) was from Gibco BRL (NY, USA). The cell adhesive Cell-Tak was from BD Biosciences (Bedford, MA, USA). Fura-2 AM was bought from AAT Bioquest (Sunnyvale, CA, USA). Rabbit anti-$\alpha_1$ adrenergic receptor antibody and TRITC-conjugated donkey anti-rabbit secondary antibody were from Abcam (Cambridge, UK). Acetochlor was dissolved in DMSO, before dilution in buffer to final indicated concentrations. Final DMSO concentration remained < 0.1% which had no effect on either basal calcium or PE-induced calcium oscillations.

**1.2 Buffers** Standard buffer for isolation of hepatocytes had the following composition (in mmol · $L^{-1}$): NaCl 118, KCl 4.7, $NaH_2PO_4$ 1.3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, D-glucose 6, L-glutamine 2, pyruvate 2.5, MEM amino acid mixture (50X) 2%, bovine serum albumin (BSA) 0.2%, pH was adjusted to 7.4, and oxygenated for 30 min before use. Perfusion buffer was made without glutamine, pyruvate, amino acid mixture or BSA. For liver perfusion and digestion with collagenase H, collagenase H 24 mg was dissolved in a standard buffer of 80 mL. For filtering out of collagenase H after hepatocytes isolation, cells were layered onto standard buffer containing BSA 2% and centrifuged.

**1.3 Isolation of rat hepatocytes** Hepatocytes were isolated from male Sprague-Dawley rats (200-400 g) bought from Experimental Animal Facilities, Military Medical Academy Beijing, as reported previously[39–41, 43]. Briefly, rat was killed and perfused via the portal vein first in calcium-free, heparin-containing (10 IU · $mL^{-1}$) buffer (warmed to 37 ℃), at a rate of 32 mL · $min^{-1}$, then in buffer containing collagenase H (0.29 g · $L^{-1}$) at a rate of 20 mL · $min^{-1}$. The perfused caudal lobe was excised and digested for a further 2 min in a shaking water-bath (37 ℃, 120 r · $min^{-1}$). Released hepatocytes were filtered, washed and re-suspended, and judged as > 95% viable by trypan blue exclusion. Only healthy cells were used for experiments as verified by robust and *persistent* calcium oscillations after PE stimulation.

**1.4 Measurement of cytosolic calcium concentration in rat hepatocytes** Hepatocyte cytosolic calcium concentration was measured as reported previously[40–41, 43–46]. Briefly, isolated rat hepatocytes were incubated with Fura-2 AM (final concentration 10 μmol · $L^{-1}$) for 30 min in a shaking water-bath (37 ℃, 50 r · $min^{-1}$), attached to the

bottom cover-slip (treated with Cell-Tak) of a Sykes-Moore perfusion chamber, and perfused at a rate of 1 mL · min$^{-1}$. Cytosolic calcium was measured in a Photon Technology International (PTI, Birmingham, NJ, USA) calcium measurement system. The excitation monochromater was DeltaRam V, with alternate excitation at 340/380 nm. Emitted fluorescence was passed through emitter (510 ± 20) nm and detected with a Hamamatsu PMT attached to an Olympus IX70 inverted fluorescence microscope. Fluorescence ratios recorded (1 ratio per min) were plotted with SigmaPlot, as representative of cytosolic calcium changes as reported before[43, 45, 47–49].

To detect any effect of acetochlor on the perifused rat hepatocytes, hepaocytes were first stimulated with the α1 adrenergic agonist PE, then acetochlor at different concentrations were added for 10 min, followed by a second dose of PE. Alternatively, hepatocytes were perfused throughout, acetochlor was then added for 15 min on top of PE stimulation. For statistical analysis, calcium spikes before, during, and after acetochor was counted and plotted as mean ± SEM (number of spikes in 15 min). Statistical significance was noted when $P < 0.05$. All experiments were repeated at least 3 times with hepatocytes from ⩾ 3 different rats killed on different days each.

**1.5 Immunocytochemical and immunohistochemical demonstration of graded α1 receptor density in isolated hepatocytes and in liver lobule** Freshly isolated rat hepatocytes were fixed in paraformaldehyde 4% for 10 min, permeabilized in 0.2% Triton X-100 for 15 min, nonspecific binding blocked in 3% BSA for 60 min, then incubated with rabbit anti-α1 adrenergic receptor antibody (1:100) at 4 ℃ for 12-14 h, TRITC-conjugated donkey anti-rabbit secondary antibody (1:100) at room temperature for 1 h. Controls were done: without primary and secondary antibodies, with primary but without secondary antibodies, without primary but with secondary antibodies, or with non-specific IgG and secondary antibody. All controls showed negative signal. Cell nucleus was visualized with Hoechst 33342. All images were taken in a laser scanning confocal microscope (Zeiss 510 Meta) under objective×63, with TRITC at $\lambda_{ex}$ 543 nm/$\lambda_{em}$ 590 nm, Hoechst 33342 at $\lambda_{ex}$ 405 nm/$\lambda_{em}$ 420 nm. The α1 adrenergic receptor fluorescence density in hepatocytes (#1-#10) was quantified with AimImageBrowser, and plotted sequentially with SigmaPlot.

For immunohistochemistry, rat liver was excised and fixed in paraformaldehyde 4% (4 ℃, 12 h), dehydrated in 30% saccharose (4 ℃, 12 h), and slices (10 μm thick) frozen cut. The slices were first incubated in 3% BSA for 60 min to block nonspecific binding, then incubated with rabbit anti-α1 adrenergic receptor antibody (1:100, 4 ℃ 12-14 h), FITC-conjugated goat anti-rabbit secondary antibody (1:100) at room temperature for 1 h. Controls were done: without primary and secondary antibodies, with primary but without secondary antibodies, without primary but with secondary antibodies, or with non-specific IgG and secondary antibody. All controls showed negative signal. Cell nucleus was stained with Hoechst 33342. Images were taken in a laser scanning confocal microscope (Zeiss 510 Meta) under objective × 20 or × 10, with FITC at $\lambda_{ex}$ 488 nm/$\lambda_{em}$ 495 nm; Hoechst 33342 at $\lambda_{ex}$ 405 nm/$\lambda_{em}$ 420 nm. The α1 receptor fluorescence intensity along lines in neighboring pericentral and periportal regions and along hepatocyte stacks in a liver lobule was quantified with AimImageBrowser and plotted with SigmaPlot.

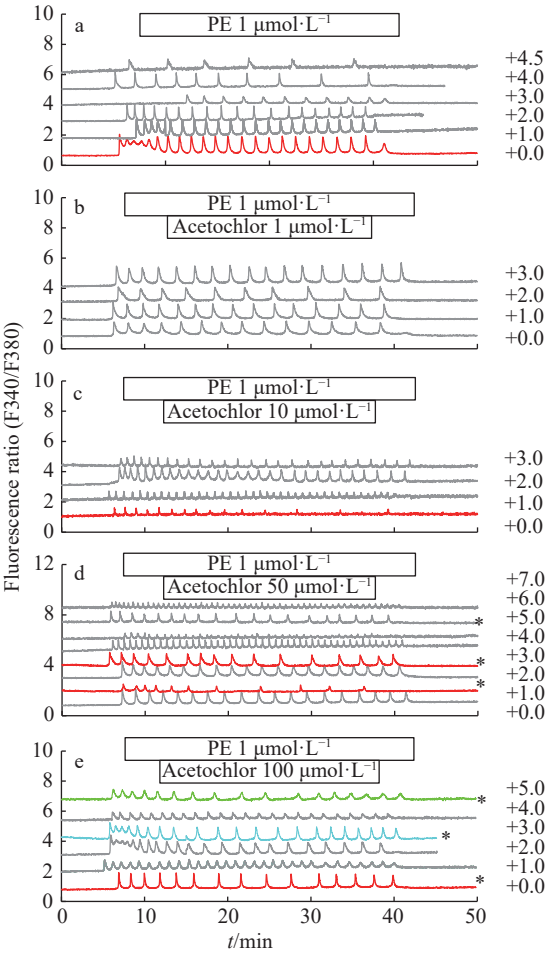
## 2 Results

### 2.1 Acetochlor inhibition of phenylepinephrine (PE)-induced calcium oscillations in selected hepatocytes

Phenylepinephrine (PE) at low concentrations induced cytosolic calcium oscillations in the freshly isolated rat hepatocytes, as reported before[39–41]. PE 1 μmol · L$^{-1}$ when added to perifused rat hepatocyte triggered regular calcium oscillations, note the intrinsic variation in oscillatory frequency in different hepatocytes (Fig. 1-a). Addition of acetochlor at 1 μmol · L$^{-1}$ had no apparent effect on PE-induced calcium oscillation (Fig. 1-b), neither did acetochlor at 10 μmol · L$^{-1}$ (Fig. 1-c). With acetochlor 50 μmol · L$^{-1}$ added, the frequency of PE-induced calcium oscillations was minimally diminished in 3 out of 8 experiments, with slight recovery after wash-out (tracings marked with *, Fig. 1-d). This inhibition was more marked with acetochlor 100 μmol · L$^{-1}$. In 3 out of 6 experiments acetochlor 100 μmol · L$^{-1}$ reduced calcium oscillatory frequency with gradual recovery after wash-out of acetochlor. The oscillatory frequency of PE-induced calcium oscillations were (0.63 ± 0.09), (0.36 ± 0.02), (0.60 ± 0.06) spikes/min before, during and after acetochlor 100 μmol · L$^{-1}$ exposure, i.e., with significant

inhibition in these hepatocytes (tracings marked with *, Fig. 1-e, $P < 0.05$, $N = 3$). Some inhibitory effect was also found in 3 other experiments, but the inhibition was less pronounced (Fig. 1-e, $N = 3$). From these experiments it is clear that acetochlor at low concentrations (1 and 10 μmol · $L^{-1}$) had no apparent effect on PE-induced cytoslic calcium oscillations, but at higher concentrations (50, 100 μmol · $L^{-1}$) acetochlor reduced the oscillation frequency, but only in selected heaptocytes.

To confirm that acetochlor inhibition shown above (Fig. 1-d, e) was not due to solvent effect, vehicle DMSO 0.1% (the highest concentration used when acetochlor was at 100 μmol · $L^{-1}$) was added, which showed no effect on PE-induced calcium oscillations (not shown).

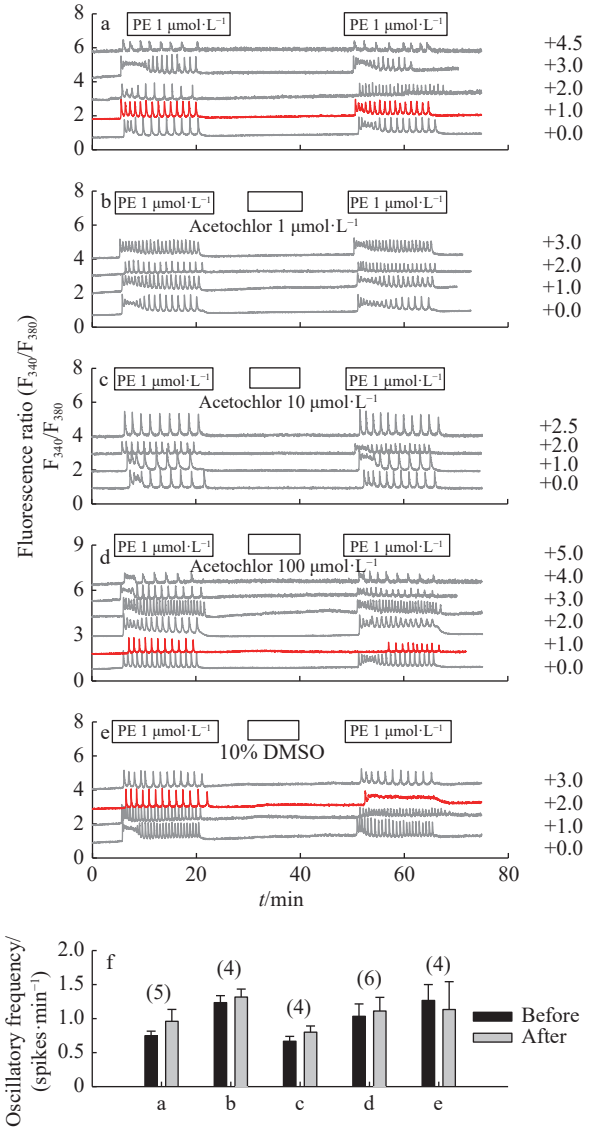



Fura-2 AM-loaded rat hepatocytes were perifused, phenylepinephrine (PE, 1 μmol · $L^{-1}$), acetochlor at indicated concentrations were added as marked by the horizontal bars. (a) Control experiment with continuous PE (1 μmol · $L^{-1}$) stimulation. Acetochlor 1 μmol · $L^{-1}$ (b), 10 μmol · $L^{-1}$ (c), 50 μmol · $L^{-1}$ (d), 100 μmol · $L^{-1}$ (e) was added during PE (1 μmol · $L^{-1}$) stimulation. Calcium tracings for each experiment are drawn together after adding the indicated values (+0.0, 1.0, 2.0, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0) to measured $F_{340}/F_{380}$ ratios. Asterisks (*) in panels (d) and (e) indicate observed acetochlor inhibition.

**Fig. 1 Inhibition by acetochlor of α1 adrenergic receptor-mediated calcium oscillations in selected hepatocytes**

**2.2 Brief acetochlor exposure showed no lasting inhibition on subsequent PE-induced calcium oscillations in the freshly isolated rat hepatocytes** Repeated stimulations of micromolar PE induced reproducible calcium oscillations in perifused rat heaptocytes. The two sequential PE challenges resulted in identical or highly similar pattern of calcium oscillations (Fig. 2-a). In between the two PE stimulations, acetochlor exposure at 1-100 μmol · $L^{-1}$ had no effect on either basal calcium (Fig. 2-b-d), or on calcium oscillations induced by a second dose of PE (Fig. 2-b-d). For comparison, DMSO at 10% did not change basal calcium concentration (Fig. 2-e), neither did it inhibit significantly the second PE stimulation in 2 experiments, but either resulted in an elevated plateau or diminished oscillations in 2 other experiments (Fig. 2-e). Therefore acetochlor at 1-100 μmol · $L^{-1}$ had no lasting effect on cytosolic calcium in hepatocytes but DMSO 10% did show minor modification of subsequent calcium oscillations (Fig. 2-f).

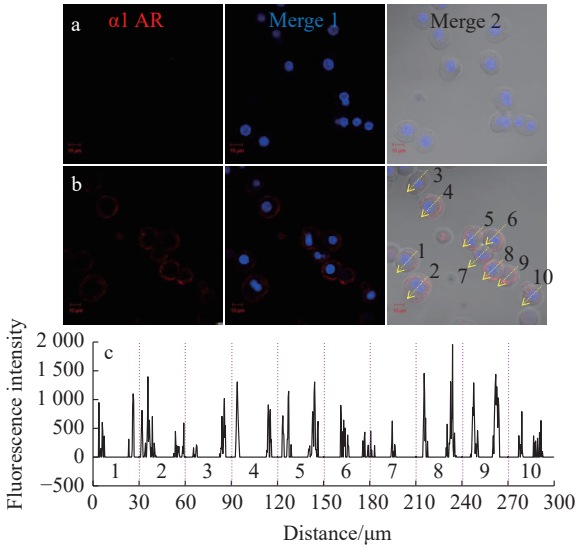
**2.3 Graded α1 adrenergic receptor density among freshly isolated rat hepatocytes** The calcium experiments above indicated that acetochlor tended to inhibit PE-induced calcium oscillations in hepatocytes which showed lower oscillatory frequency after PE stimulation at 1 μmol · $L^{-1}$ (Fig. 1). Lower frequency of calcium oscillation could either be due to low PE concentration, or due to low density of α1 adrenergic receptor. Since PE concentration used was the same, we decided to examine possible variations in α1 adrenergic receptor density in the freshly isolated rat hepatocytes by immunocytochemsitry. Fig. 3 reveals varied α1 adrenergic receptor density in different hepatocytes. Control experiments indicated that without anti-α1 adrenergic receptor antibody, no staining was found (Fig. 3-a). The α1 adrenergic receptor was mainly localized on the plasma membrane in the isolated hepatocyets with the clearly defined nuclear and cytosolic regions without staining (Fig. 3-b). The receptor density was markedly different among isolated hepatocytes (Fig. 3-b). Of those hepatocytes shown in Fig. 3-b, the receptor density was the highest in cell number 8, and lowest in cell number 7, with the rest in between: 8 > 9 > 2 > 5 > 4 >




Fura-2 AM-loaded rat hepatocytes were perifused, PE (1 μmol · $L^{-1}$), acetochlor (1, 10, 100 μmol · $L^{-1}$), or 10% DMSO were added as indicated by the horizontal bars. (a) Control: 2 sequential PE 1 μmol · $L^{-1}$ stimulations. Acetochlor 1 μmol · $L^{-1}$ (b), 10 μmol · $L^{-1}$ (c), 100 μmol · $L^{-1}$ (d), 10% DMSO (e) were added in between 2 PE 1 μmol · $L^{-1}$ stimulations. Calcium tracings for each experiment are drawn together after adding the indicated values (+0.0, 1.0, 2.0, 2.5, 3.0, 4.0, 4.5, 5.0) to measured $F_{340}/F_{380}$ ratios. (f) The frequencies of calcium oscillation in panels (a-e) were analyzed. In each case (a, e) frequency of first PE stimulation ("Before") was not significantly different from that of second PE stimulation ("After"). No statistical significance was detected.

**Fig. 2　Acetochlor or DMSO had no effect on sequential stimulation of α1 adrenergic receptor by PE in the freshly isolated rat hepatocytes**

1 > 3 > 6 > 10 > 7 (Fig. 3-c). Note that in the fluorescent density plot, each hepatocyte is indicated by 2 major cluster peaks, representing the opposing plasma




Each panel is composed of micrographs of α1 adrenergic receptor fluorescence (red), nuclear fluorescence (blue), and bright-field merged images. Freshly isolated rat hepatocytes were fixed, incubated without (a) or with (b) rabbit anti-α1 adrenergic receptor, then with TRITC-conjugated donkey anti-rabbit secondary antibody (red), and double stained with nuclear stain Hoechst 33 342 (blue). The α1 receptor fluorescence intensity along the dotted lines for each hepatocyte was quantified with software AimImageBrowser, and plotted sequentially from hepatocyte # 1 to # 10 with SigmaPlot (c). Confocal images were obtained in a Zeiss laser scanning microscope (Zeiss LSM 510 META) under objective 63 x / 1.40 oil. Scale in image is 10 μm. Images shown are representative of 3 identical experiments ($N = 3$).

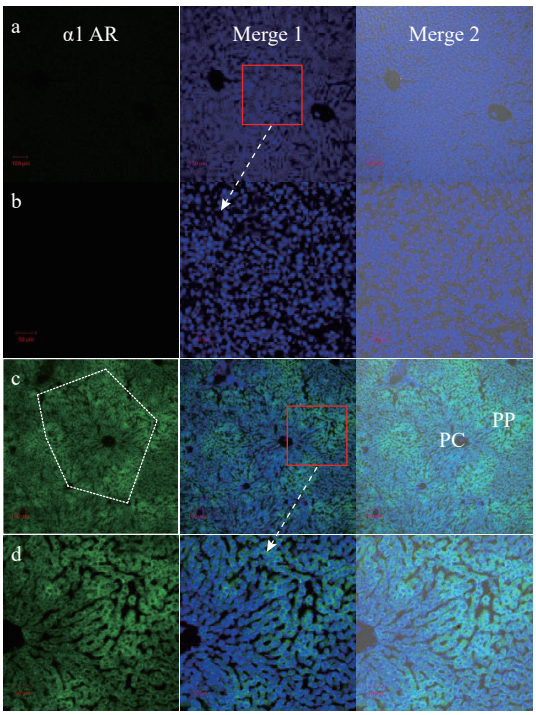
**Fig. 3　Graded α1 adrenergic receptor density in freshly isolated rat hepatocytes**

membrane patches along the scan line (Fig. 3-c).

**2.4　A clear adrenergic α1 receptor density pattern in the liver lobule**　To correlate receptor density with *in situ* localization of hepatocyte, immunohistochemistry was performed in thinly-cut liver slices. In such slices, each lobule was found to be composed of hepatocytes arranged radially, surrounding neighboring portal triad or central vein. The α1 adrenergic receptor density among neighboring hepatocytes in the liver lobule in frozen-cut liver slices showed typical graded receptor density (Fig. 4). Control experiments without added primary antibody but with secondary antibody did not show any staining for the α1 adrenergic receptor (Fig. 4-a, 4-b). The lower panel micrographs (Fig. 4-b, 4-d) are the expanded portions from the upper panels (Fig. 4-a, 4-c). The α1 adrenergic receptor density varied depending on the hepatocyte location in the lobule (Fig. 4-c, 4-d). The highest density was found in the periportal (PP) region (marked by the

dashed lines in Fig. 4-c), with receptor density gradually decreasing towards the pericentral (PC) region (Fig. 4-c, 4-d).

Such graded α1 adrenergic receptor density was analyzed and presented quantitatively in Fig. 5-A and 5-B. The micrograph in Fig. 5-Aa is taken from the rectangular part in Fig. 4-c. Tracings 1-6 in Fig. 5-Ab show that in each and every direction (lines 1-6) starting from the central vein (pericentral, PC), α1 adrenergic receptor density increased gradually in a pericentral-to-periportal (PC-to-PP) direction, reaching a maximum at the periportal (PP) triad. A line drawn between 2 central veins (Fig. 5-Aa, line 7) showed an overall "mountain peak" structure (Fig. 5-Ab, tracing 7).



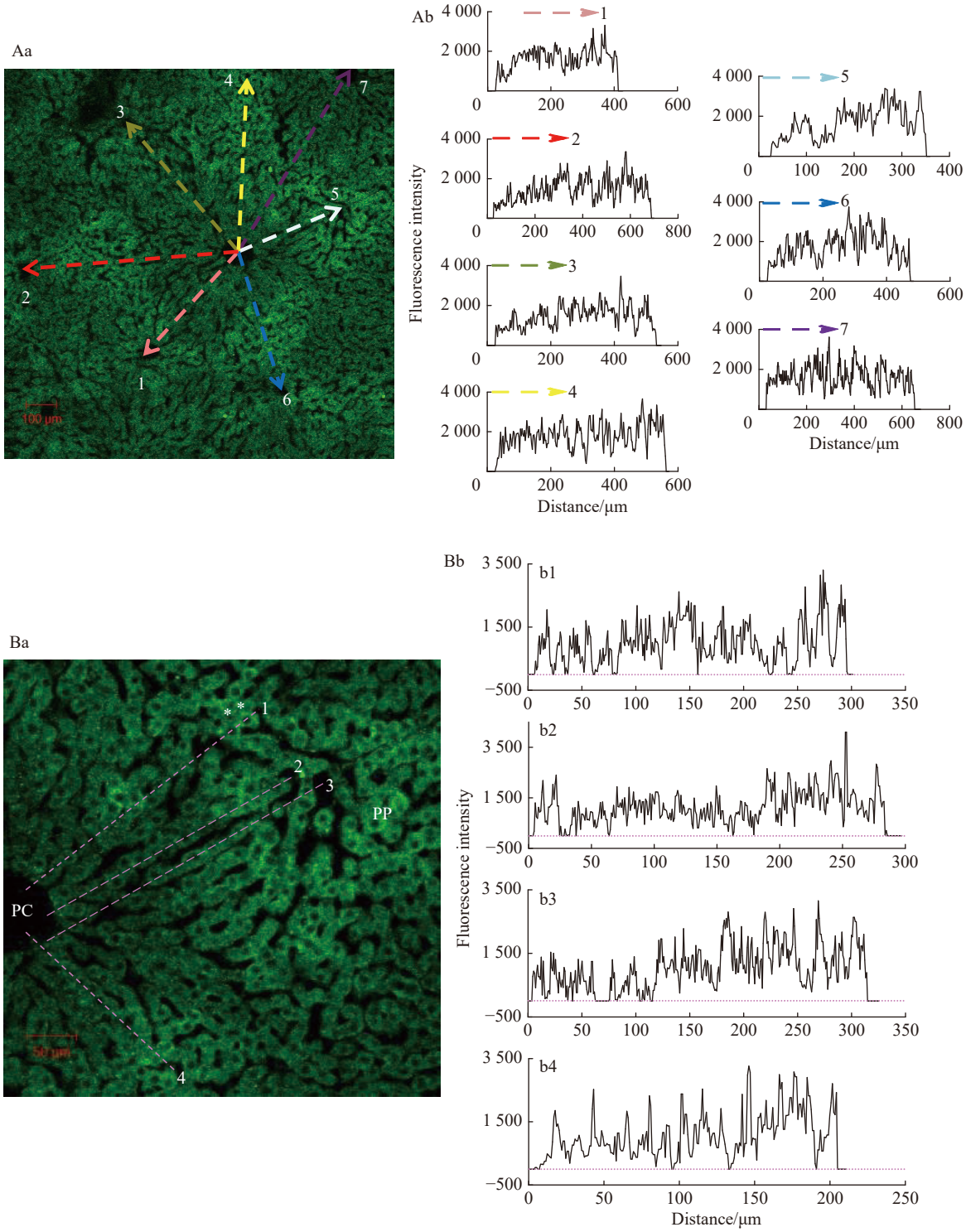

Each panel is composed of α1 adrenergic receptor fluorescence, merged α1 adrenergic receptor/nuclear fluorescence (merge 1), and merged α1 adrenergic receptor/nuclear fluorescence/bright field (merge 2) images. Frozen rat liver slices (10 μm thick) were cut, incubated without (a, b) or with (c, d) rabbit anti-α1 adrenergic receptor antibody, then with FITC-conjugated goat anti-rabbit secondary antibody (green), and double stained with Hoechst 33342 (blue). Images were taken in a laser scanning confocal microscope (Zeiss LSM 510 META) under objectives (in magnification/numerical aperture) 10 x/1.40、20 x/1.40, scales shown are 50 or 100 μm. Panels b and d were taken from Panels a and c respectively as outlined by the red squares. Images shown are representative of 4 identical experiments ($N = 4$).

**Fig. 4 Graded density distribution of $\alpha_1$ adrenergic receptor in rat liver slice**

Fig. 5-Ba (taken from Fig. 4-d) shows clear stacks of hepatocytes. Line scans (lines 1-4) along such hepatocyte stacks indicate that the α1 adrenergic receptor density (fluorescence intensity) was the highest in hepatocyte at the periportal end of the stack and lowest in hepatocyte at the pericentral end of the stack (Fig. 5-Bb, tracings 1-4). Distinct single hepatocyte profiles could be clearly recognized in such line scan tracings in that each hepatocyte is composed of 2 peaks representing that the scan line crossing the opposing plasma membranes; see the asterisks in both micrograph and tracing b1, for example. Although the reason for such graded α1 adrenergic receptor density is not clear, it is obvious that nutrient/hormone gradients are likely to play a major role.

## 3 Discussion

It has been found in the present work that when acetochlor was added to PE-stimulated rat hepatocytes, low acetochlor concentrations (1, 10 μmol · $L^{-1}$) had no effect on PE-induced calcium oscillations, but higher concentrations (50, 100 μmol · $L^{-1}$) showed reversible inhibition in selected hepatocytes which showed lower oscillatory frequency. Acetochlor (1-100 μmol · $L^{-1}$) had no effect on basal cytosolic calcium, nor did it have any effect on calcium oscillations induced by a second dose of PE. Acetochlor inhibition was reversible and the inhibition was seen only in selected hepatocytes which showed lower oscillatory frequencies. The selective inhibition was interesting and could be due to lower α1 adrenergic receptor density levels in hepatocytes from the pericentral region of the liver lobule. The increasing α1 adrenergic receptor density from pericentral-to-periportal portion of the liver lobule denotes that hepatocytes close to the central vein showed the lowest receptor density whereas hepatocytes close to the portal triad (hepatic artery, portal vein, bile duct) showed the highest receptor density. Lower receptor density results in lower frequency of calcium oscillation after receptor activation, therefore is more susceptible to acetochlor inhibition. These data together indicate that acetochlor had reversible inhibitory effect on hepatocyte α1 adrenergic receptor, but only in selected hepatocytes probably corresponding to the central vein region in the liver lobule. Future live liver slice or *in situ* calcium imaging likely will explicitly confirm such




A. Fluorescence quantification of α1 adrenergic receptor distribution in rat liver lobules. The main image is from Fig. 4-c，fluorescence intensity was scanned as indicated by the colored arrows，from pericentral（PC）to periportal（PP）regions. Purple arrow transcends two neighboring pericentral regions. Fluorescence intensity was quantified with AimImageBrowser，and plotted with SigmaPlot. Data shown are typical of *N* identical experiments（$N=6$）. B. Fluorescence quantification of α1 adrenergic receptor distribution in hepatocyte stacks of the rat liver lobule. The main image is from Fig. 4-d，fluorescence intensity was scanned as indicated by the numbered dashed lines，in the pericentral（PC）-to-periportal（PP）direction. Fluorescence intensity was quantified with AimImageBrowser，and plotted with SigmaPlot. Note the 2 white asterisks at the periportal end of the dashed line 1 in the micrograph and the last 3 cluster peaks between 250 - 300 μm in tracing b1 denote the location of 2 neighboring periportal hepatocytes. The thin dashed pink lines in tracings b1-b4 indicate blank area with zero fluorescence.

**Fig. 5　A pericentral-to-periportal gradient of α1 adrenergic receptor density in rat liver lobule**

findings.

The α1 adrenoceptor is a major receptor involved in calcium signaling[40−42], and plays a pivotal role in hepatocyte metabolism, such as hepatic glucose production[42, 50−51] and liver regeneration[52]. Therefore acetochlor interference of α1 adrenergic receptor calcium signaling bears special significance, especially when *in vivo* or *in situ* acetochlor concentration reached high levels (18.61 μmol · $L^{-1}$)[36].

Although toxic effect of acute high acetochlor concentration has not been systematically examined, chronic effect of nanomolar acetochlor has been well investigated. Nanomolar acetochlor (10 nmol · $L^{-1}$) has been found to influence tadpole metamorphosis by increasing thyroid hormone receptor expression and to accelerate T3-induced metamorphosis in *Xenopus laevis* tadpoles[34−35].

Long-term (21 d) acetochlor exposure (0.07, 0.74, 7.41 nmol · $L^{-1}$) has been found to enhance liver expression of the thyroid hormone receptor α in adult rare minnow (*Gobiocypris rarus*), acetochlor at lower concentration (0.07 nmol · $L^{-1}$) enhanced but at higher concentrations (0.74, 7.41 nmol · $L^{-1}$) diminished brain thyroid hormone receptor α expression[31]. Acetochlor feeding (5 mL · $kg^{-1}$ in sunflower seed oil, 20% $LD_{50}$ for 6 d) increased estrogen-binding capacity in uniparous female rat[32]. Acetochlor feeding (7.58, 15.36 mg · $kg^{-1}$ · $d^{-1}$, 6 d) also increased uterine nuclear estrogen receptor density[33]. Significant changes in gene (*betaine homocysteine methyltransferase*, *chemotaxin LECT2*, and *apolipoprotein E1*) expression in liver have been detected after 62 d exposure to a mixture of herbicides (total concentration 4 μg · $L^{-1}$) including acetochlor (0.5 μg · $L^{-1}$ or 1.85 nmol · $L^{-1}$) in the European flounder *Platichthys flesus*[53].

Early toxicological work at mega doses of acetochlor (peri-maximum tolerated dose, 1 000 mg · $kg^{-1}$ or 1 000 ppm in diet) found that acetochlor was not genotoxic but was epigenetically carcinogenic, and identified three susceptible toxicological targets: human lymphocytes, rat (but not mouse) nasal epithelium, and rat liver[54]. The active group in the acetochlor molecule was found to be the chloroacetyl (— $COCH_2Cl$) substituent, which targets sulfhydryl (–SH) groups in glutathione (GSH) and protamine[54−55]. The targeted action of acetochlor was highlighted further by the fact that rat sperms were remarkably resistant to acetochlor toxicity; even when rats were treated with maximum or supra-maximum tolerated doses, no mutagenic or even histopathological effects were observed[55].

Acetochlor administration at maximum tolerated dose over extended time period (2 year feeding) induced nasal adenoma in the rat[54, 56]. Nasal epithelium accumulation of an acetochlor metabolite sulfoxide quinine-imine after oral doses, and its covalent-bonding to Cys free sulfhydryl groups (Protein-Cys-SH) were noted to be important for adenoma induction[56]. Blood plasma analysis indicated that hepatocyte metabolite acetochlor sulfoxide was the major circulating metabolite available to nasal epithelium, where it was further metabolized to the reactive sulfoxide quinine-imine[56].

Supra-maximum tolerated dose also induced occasionally thyroid tumors in the rat, which were associated with increased levels of thyroid stimulating hormone (TSH) and thyroid weight, but decreased thyroid hormone T3[54, 57]. This could be due to the anti-thyroid effect of acetochlor. It was found that acetochlor enhanced uridine diphosphate glucuronosyltransferase (UDPGT) activity in *liver* therefore increased hepatic thyroid hormone metabolism and excretion; lowered thyroid hormone level led to persistent stimulation of thyroid follicular cell therefore thyroid hyperplasia and carcinogenesis[57].

Possible herbicide effect on calcium signaling has not been investigated in detail before. Herbicide 2, 4-dichlorophenoxyacetic acid has been reported to have no effect on cytosolic calcium in the isolated CD4+ T lymphocytes, but when lymphocytes were previously activated with soluble anti-CD3 antibody, 2, 4-dichlorophenoxyacetic acid (≥ 100 μmol · $L^{-1}$) stimulated irreversible and persistent (1 to 24 h) calcium increase[58]. Such calcium increases may be mediated by 2, 4-dichlorophenoxyacetic acid-stimulated production of hydrogen peroxide[58].

The acute effect of acetochlor has been examined in neurons. Acetochlor (100, 200, 400, 800 μmol · $L^{-1}$) exposure over extended time periods (24, 50 h) was found to decrease the amplitude of field stimulation-elicited compound action potential in sciatic nerve of the frog *Rana ridibunda*, both exposure time- and concentration-dependently[59]. At physiological pH the $IC_{50}$ was found to be 220 μmol · $L^{-1}$, the dose-response curve being so steep

that acetochlor at 100 μmol · $L^{-1}$ was without any effect[59]. At pH 3.3, the sciatic nerve was severely compromised, $IC_{50}$ was found to be 770 μmol · $L^{-1}$ but again acetochlor at 600 μmol · $L^{-1}$ was without any effect[59]. In shorter time incubations the neurons survived better. The shortest time point examined was 1 h, acetochlor at ≤ 400 micro mol · $L^{-1}$ had no effect, but at 800 μmol · $L^{-1}$ only 65% nerve vitality remained[59]. In this interesting work, no molecular mechanisms (ionic channel involvement or nerve cell plasma membrane permeability changes) were examined. Acetochlor effect on cell vitality has also been examined in cell lines Vero, HepG2, MCF7, cytotoxic $IC_{50}$ was found to be 57, 116, and 231 μmol · $L^{-1}$ (15.6, 32.1, 62.5 μg · $mL^{-1}$) respectively[60]. It may be noted that $LC_{50}$ (in 48 h) of acetochlor in invertebrates has been found to be in the mmol · $L^{-1}$ range (0.47-4.37 mmol · $L^{-1}$)[61−62].

Acetochlor (10, 100, 1 000 μmol · $L^{-1}$) stimulated the multidrug resistance (MDR) type 1 (MDR1) (an ATP-binding cassette or ABC transporter) ATPase activity with an $EC_{50}$ of 25 μmol · $L^{-1}$, inhibited calcein efflux with an $IC_{50}$ > 100 μmol · $L^{-1}$, but had little effect on other ABC transporters (MRP1, MRP2, BCRP)[1]. Acetochlor stimulation of MDR1 activity sensitized MDR1-K562 cells to cytostatic paclitaxel[1].

Prolonged acetochlor exposure (for many h) had different effects. Acetochlor up to 10 μmol · $L^{-1}$ (48 h) had no effect on the viability of human breast cancer cell lines MCF-7, MCF-10A, MDA-MB-231[63]. But acetochlor at 1 mmol · $L^{-1}$ induced significant (6%) human erythrocyte hemolysis (in 24 h)[64]. Acetochlor exposure (25-800 μmol · $L^{-1}$) to human lung carcinoma cell A549 resulted in reactive oxygen species generation (in 2-3 h) and apoptosis (in 24-120 h)[65]. Acetochlor was found to have little cytotoxicity in fibroblasts (AS52) at 10 μmol · $L^{-1}$, although at 100 μmol · $L^{-1}$ significant toxicity was detected; acetochlor at both 4 and 40 μmol · $L^{-1}$ increased the proportion of cells at G1 stage of the cell cycle, with a corresponding decrease in G2 phase[66].

As mentioned above the most susceptible cell type in *in vivo* studies in rat has been found to be hepatocytes[54]. The hepatocyte toxicity of acetochlor (at 100, 200, 400, 800, 1 600 μmol · $L^{-1}$) has been examined by measuring lactate dehydrogenase (LDH) leakage from intact hepatocytes. It was found that acetochlor at 100 μmol · $L^{-1}$ had no effect after incubation with isolated rat hepatocytes for 2 h, cytotoxic $LC_{50}$ (50% toxicity) was 706 μmol · $L^{-1}$[2]. In two lots of cryo-preserved human hepatocytes, acetochlor at 200 or 400 μmol · $L^{-1}$ had no effect after 2 h incubation; the cytotoxic $LC_{50}$ was found to be 587, 1 348 μmol · $L^{-1}$ respectively[2]. These data clearly indicated that acetochlor was quite non-toxic towards hepatocytes even at 100 μmol · $L^{-1}$ over an extended incubation period (2 h). Although acetochlor (1-100 μmol · $L^{-1}$) was non-toxic for freshly isolated rat hepatocytes, it did inhibit PE-induced calcium oscillations reversibly in selected hepatocytes (Fig. 1).

The present work found that not all hepatocytes were susceptible to acetochlor inhibition. Reversible inhibition by acetochlor was seen only in hepatocytes showing more sparse calcium oscillations (Fig. 1), likely due to lower α1 adrenergic receptor density (Fig. 3, 4). Since hepatocytes with sparse calcium oscillations are likely pericentral (Fig. 4, 5) therefore more distant to the circulating portal blood, in whole organisms pericentral hepatocytes may be less affected than periportal hepatocyte by circulating acetochlor. The fact that α1 adrenergic receptor is present in the highest density in periportal region (Fig. 4, 5) is consistent with reports that calcium waves always start from the periportal region to spread to pericentral hepatocytes in the liver[67−68]. To our best knowledge, the present work is the first fluorescent immunohistochemical report of a pericentral-to-periportal gradient of α1 adrenergic receptor density (Fig. 4, 5). In the present work it was not possible to correlate hepatocyte receptor density in frozen cut liver slice (10 μm thick) directly with calcium oscillation. In the future it will be interesting to investigate, in live liver sections, to correlate modulated calcium signaling with receptor density, and possibly also with density of other signaling proteins.

Although brief incubation with acetochlor 100 μmol · $L^{-1}$ had no effect on basal calcium concentration in the freshly isolated rat hepatocytes (Fig. 2) and showed rather reversible inhibition of PE-induced calcium oscillations (Fig. 1), acetochlor 100 μmol · $L^{-1}$ has been reported to increased cytosolic calcium concentration markedly with extended incubation period of 24 h in the human liver carcinoma cell line HepG2[69]. Acetochlor 20 mg · $L^{-1}$ (74.1 μmol · $L^{-1}$) was found to induce apoptosis and related signaling in a large majority of HepG2 cells over a

24 h period[70]. Continued monitoring of cytosolic calcium concentration over extended time period (24 h or longer) might give some details as to the time course of calcium changes, it must be born in mind that acetochlor might not be cleared out of the body very quickly in the exposed patients.

As mentioned above, long-term exposure (2 year feeding) of acetochlor in rat leads to nasal tumor, largely due to persistent presence in nasal epithelium of a sulfoxide intermediate. Hydroxylation of the phenyl ring of the sulfoxide intermediate to quinine-imine was important for hyperplasia induction in nasal epithelium[56]. This reactive quinine-imine/quinonimine could react covalently with the sulfhydryl groups in protein side chain to induce protein modification[56, 71]. Metabolite studies in rat liver microsomes found that acetochlor was metabolized sequentially to 2-cholor-N-(2-methyl-6-ethylphenyl)acetamide (CMEPA), 2-methyl-6-ethylaniline (MEA), and dialkylbenzoquinone[72]. Whether adrenoceptor Cys thiols would play any role during brief incubation with acetochlor in perfused heaptocytes is not known at present. Free thiol oxidative modulation of receptor may play a role in acetochlor inhibition of the α1 adrenoceptor signaling.

In conclusion, acetochlor at high concentrations inhibits α1 adrenoceptor-mediated calcium signaling in selected hepatocytes, which may have implications for occupational exposure and acute poisoning. This acute effect on α1 adrenoceptor adds to the well-recognized long-term effect on thyroid hormone receptor and estrogen receptor.

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# 除草剂乙草胺抑制大鼠肝脏细胞肾上腺素能受体所介导的胞浆钙振荡

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**摘要**　田间除草剂乙草胺施用之后非常容易进入地表水和地下水, 河流溪水中乙草胺浓度可达纳摩尔水平, 从而对水生生物产生长期影响. 因职业关系而产生的皮肤暴露和吸入, 可导致人血液乙草胺浓度达到微摩尔水平. 对乙草胺体内动力学的研究, 发现肝脏是乙草胺毒理作用的主要靶器官. 已知在肝脏细胞多种生理功能中, 钙离子发挥重要作用. 本文研究在新鲜分离的大鼠肝脏细胞, 乙草胺对肾上腺素能受体所介导胞浆钙振荡的可能影响. 实验发现低浓度乙草胺(1、10 μmol·$L^{-1}$)对苯丙肾上腺素所诱导钙振荡没有影响, 但是高浓度(50、100、200 μmol·$L^{-1}$)乙草胺在有些肝脏细胞可逆性抑制苯丙肾上腺素所诱导的胞浆钙振荡. 在苯丙肾上腺素 2 次串联刺激之间短暂加入乙草胺(1、10、100 μmol·$L^{-1}$), 乙草胺对肝脏细胞基础钙浓度没有影响, 也不影响第 2 次苯丙肾上腺素刺激所引发胞浆钙振荡. 细胞免疫化学研究发现在新鲜分离的大鼠肝脏不同细胞, α1 肾上腺素能受体密度存在明显差异. 对固定的大鼠肝脏切片进行组织免疫化学检测, 发现 α1 肾上腺素能受体在肝脏小叶的密度梯度分布: α1 肾上腺素能受体密度从中央静脉方向到门脉方向从低到高逐渐增加. 这种 α1 肾上腺素能受体的密度梯度分布, 可能是乙草胺选择性抑制苯丙肾上腺素刺激分离大鼠肝脏细胞所诱发钙振荡的原因. 本文数据揭示, 在急性皮肤暴露或乙草胺中毒后可达到的血液浓度, 短期施加乙草胺, 可一过性抑制肝脏 α1 肾上腺素能受体所介导的胞浆钙振荡. 长期乙草胺暴露, 可能对肝脏细胞正常钙信号系统产生持续性不良影响.

**关键词**　乙草胺; 肾上腺素能受体; 钙振荡; 肝脏细胞

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