The calibration curves were constructed with $1/x^2$ weighting and the regression coefficients were greater than 0.99.

2.7. Other analyses

Protein concentrations were determined with BCA assay (Pierce) based on bovine serum albumin standard. Data are presented as mean ± S.D. All enzymatic assays were repeated three times with the same microsomal preparation. Statistical tests were performed to compare means (Student's t-test) or correlations (Spearman). In all cases, significant differences were assumed when p-values were less than 0.05.

3. Results

3.1. Ontogenic expression of human carboxylesterases HCE1 and HCE2 by RT-qPCR

In this study, we first evaluated the expression patterns of human carboxylesterases HCE1 and HCE2 in various age groups. A total of 104 RNA samples were analyzed including 48 samples from fetuses (82–224 gestation days), 34 from children (0 days–10 years of age) and 22 from adults (≥18). The levels of HCE1 and HCE2 mRNA were determined with RT-qPCR, and the results are summarized in Fig. 1 and Table 2. Overall, the adult group had the highest levels of HCE1 and HCE2 mRNA, and the fetal group had the lowest levels for both enzymes. Based on the values of the means, the adult group expressed HCE1 at levels 319-fold higher than the fetal group, and ~50% higher than the child group (Fig. 1 and Table 2). Likewise, the adult group expressed HCE2 at levels 55-fold higher than the fetal group and ~40% higher than the child group. In all cases, the differences among various age groups were statistically significant (Fig. 1). It should be noted that two different sets of Y axial values were used in Fig. 1 to accommodate the large difference in the mRNA levels between the fetal and the other two groups.

In addition to the large difference among various age groups (inter-group), a large inter-individual variability was detected within a group. The fetal group, for example, showed a 431-fold difference (ratio between the maximum and the minimum) in HCE1 mRNA with a coefficient of variation (CV) of as high as 172% (Table 2). The child and adult groups, on the other hand, varied less in HCE1 mRNA with a 218- and 12-fold difference, respectively (Table 2). Similarly, the levels of HCE2 mRNA varied in all age groups, however, the overall variability was much less than HCE1 mRNA. The variation in HCE2 mRNA

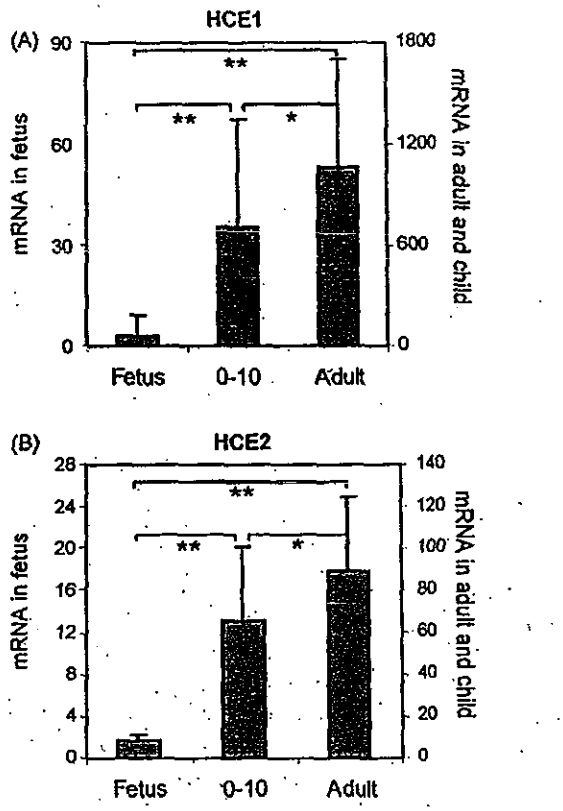

Fig. 1 – Levels of HCE1 and HCE 2 mRNA in the adult, child and fetal groups. Total RNAs were subjected to RT-qPCR analysis for the level of HCE1 mRNA (A) and HCE2 mRNA (B) by Taqman probes as described in Section 2. The adult group contained 22 samples, the child group contained 34 samples and the fetal group contained 48 samples. The signals from each target were normalized based on the signal from PolII and expressed as relative levels among all samples. The data are presented as mean ± S.D. (*) Statistical significance at $p < 0.05$, (**) statistical significance at $p < 0.001$.

was the same in the fetal and child groups and higher than that in the adult group (21- versus 4-fold) (Table 2).

We next examined whether the expression of carboxylesterases is age-related within a group. The levels of mRNA were plotted against age or gestation days, and the correlation coefficients were computed. The adult group showed no clear correlation for either HCE1 or HCE2 (data not shown). In the fetal samples, the level of HCE2 but not HCE1 mRNA was

Table 2 – Relative mRNA levels of carboxylesterases *HCE1* and *HCE2* in fetuses, children (0–10 years old) and adults (≥18).

Group	n	Minimum	Maximum	Variability	Mean	S.D.	CV (%)
Fetus-HCE1	48	0.07	30.15	431 (fold)	3.32	5.70	172
Child-HCE1	34	12.18	2657.77	218	711.47	638.51	90
Adult-HCE1	22	225.50	2659.61	12	1059.59	644.92	61
Fetus-HCE2	48	0.20	4.28	21	1.63	1.36	91
Child-HCE2	34	7.00	148.78	21	65.82	34.90	53
Adult-HCE2	22	39.89	168.66	4	89.87	34.83	39

Abbreviations: S.D.: standard deviation; CV: coefficient of variation.

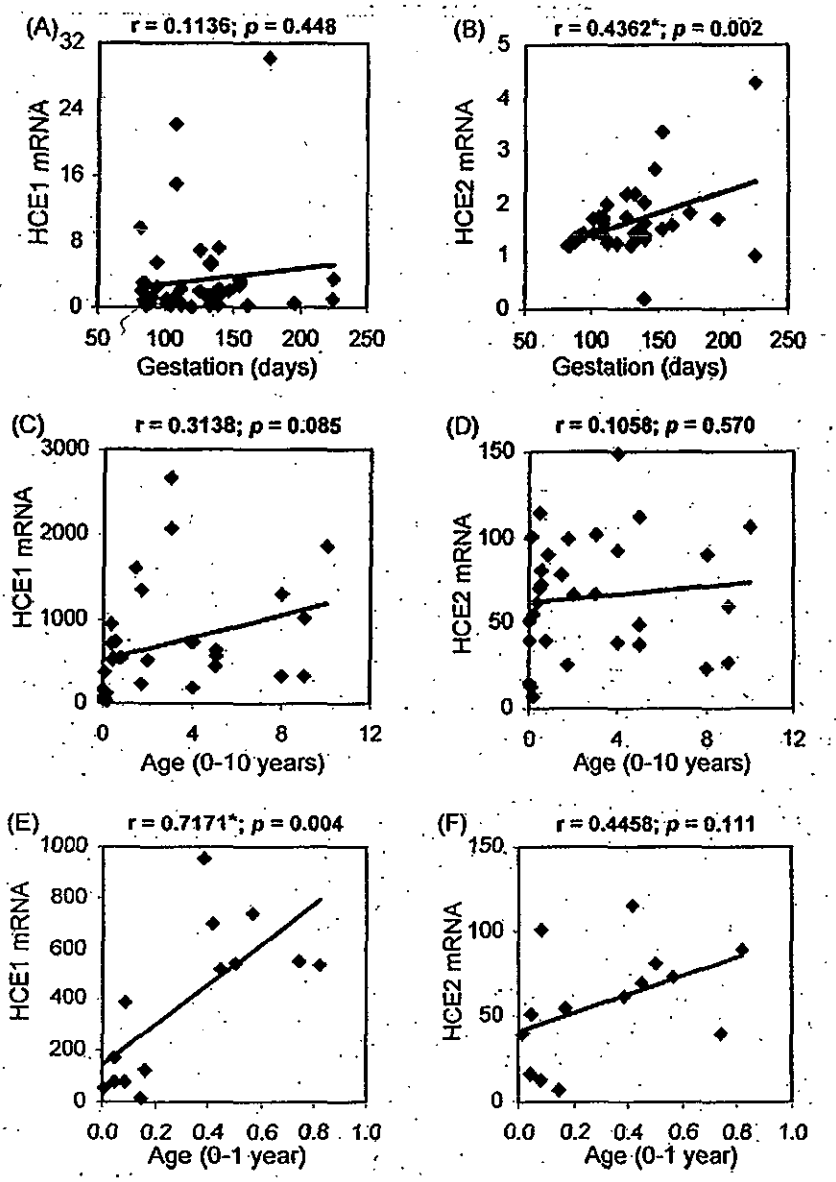

Fig. 2 – Age-related expression of HCE1 and HCE2. The data from Fig. 1 were plotted against age with SPSS version 16. (A) Correlation of HCE1 mRNA with gestation days. (B) Correlation of HCE2 mRNA with gestation days. (C) Correlation of HCE1 mRNA with age in the child group (0–10 years old). (D) Correlation of HCE2 mRNA with age in the child group (0–10 years old). (E) Correlation of HCE1 mRNA with age in the sub-child group (0–1-year-old). (F) Correlation of HCE2 mRNA with age in the sub-child group (0–1-year-old), (*) statistical significance ($p < 0.05$).

significantly correlated with age (Fig. 2A and B). In contrast, the child group showed better correlation with age on HCE1 than HCE2 mRNA, although neither correlation reached the level of statistical significance (Fig. 2C and D). To gain additional information on age-related expression, correlation analysis was performed on the samples from donors under one year old (0–1 year). In this sub-group, much improved correlation was observed on both HCE1 and HCE2 with a p-value of 0.004 and 0.111, respectively (Fig. 2E and F).

3.2. *Inter-individual variability in carboxylesterase proteins and oseltamivir hydrolysis in the child group*

We next examined whether the levels of carboxylesterase mRNA reflected the levels of corresponding proteins. RNA-microsome matched samples from 11 available pediatric-aged subjects were evaluated. Fetal matched samples were not tested because of low mRNA expression and low activities towards marker substrates (described below). No RNA-microsome matched samples were available for the adult group. The microsomal samples were first analyzed by Western immunoblotting with antibody against HCE1 or HCE2, and the immunostaining intensities were quantified by the KODAK 1D Image Analysis Software. As shown in Fig. 3A, all samples contained HCE1 and HCE2 proteins, but the relative abundance varied markedly. In particular, one donor (lane 6) showed extremely low levels of both carboxylesterases (Fig. 3A). Based on the immunostaining intensities, HCE1 protein varied by ~100-fold, and HCE2 protein varied by ~20-fold. The inter-individual variability, however, was decreased

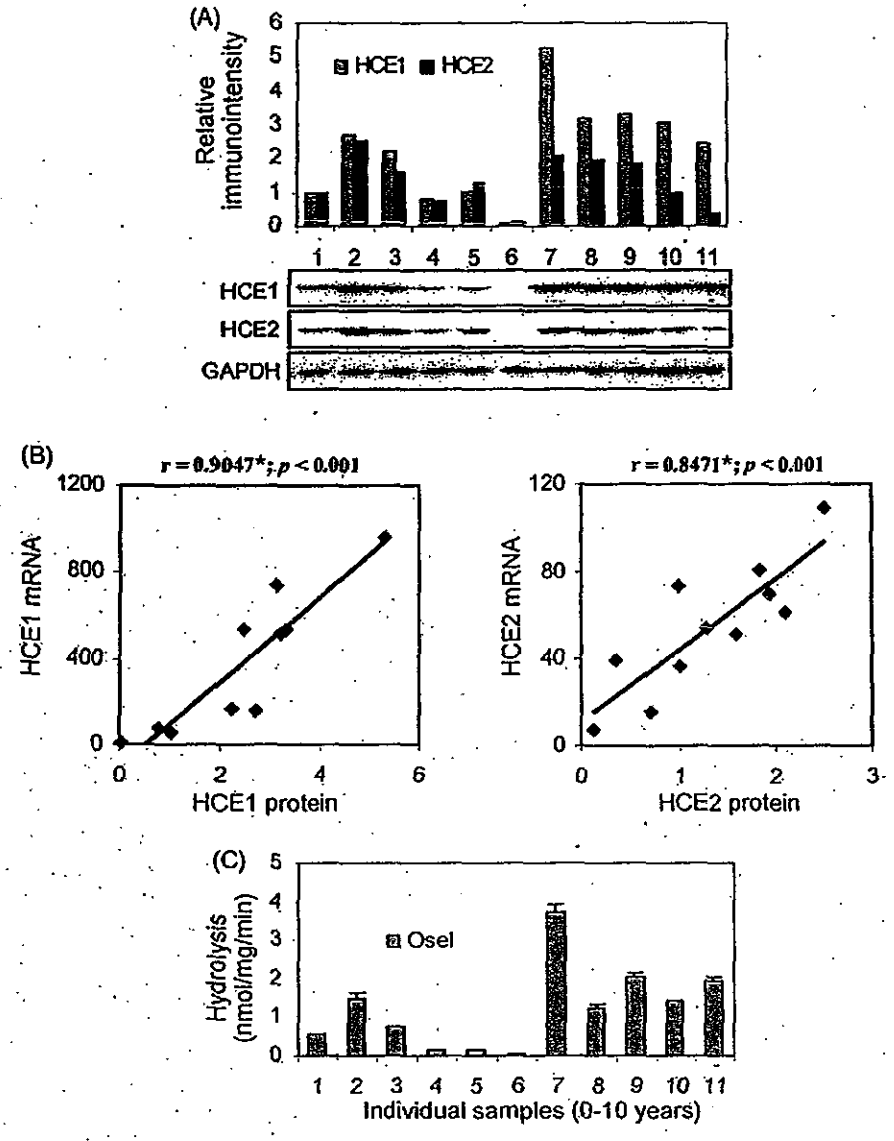

Fig. 3 – Individual variation of HCE1 and HCE2 proteins and oseltamivir hydrolysis in the child group. (A) *Western analyses.* Microsomes (1.5 μg) were resolved by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blots were incubated with an antibody against HCE1, HCE2 or GAPDH and chemiluminescent substrate. The signal was captured by a KODAK Image Station 2000 and the relative intensities were quantified by the KODAK 1D Image Analysis Software. (B) *Correlation analyses.* The immunointensities of HCE1 and HCE2 were plotted against the levels of respective mRNA. The correlation coefficients and evaluations on statistical significance were performed with SPSS version 16. (*) Statistical significant ($p < 0.001$). (C) *Oseltamivir hydrolysis by individual liver samples of the child group.* Microsomes (20 μg) were incubated with oseltamivir (200 μM) at 37 °C for 10 min, and the formation of oseltamivir carboxylate was detected by LC–MS/MS. Data were assembled from three independent experiments with two injections of each experiment.

to ~8-fold for both carboxylesterases when the data-point (lane 6) was eliminated. For both HCE1 and HCE2, the mRNA levels were correlated significantly with the levels of respective proteins ($p < 0.001$) (Fig. 3B). The HCE1 matched samples had a correlation coefficient of 0.9047, and the HCE2 matched samples had a slightly lower correlation coefficient (0.8471) (Fig. 3B).

The large individual variability in carboxylesterase protein pointed to the possibility of marked differences in the metabolism of therapeutic agents and other xenobiotics. To directly test this possibility, the anti-influenza viral agent oseltamivir (an ester prodrug) was incubated with individual donor samples (Fig. 3A), and the hydrolysis was monitored. As shown in Fig. 3C, all samples hydrolyzed this anti-viral agent, and the overall hydrolysis varied by 127-fold. Such a large inter-individual variability was in agreement with the variation in the abundance of HCE1 (Fig. 3A), which has been shown to catalyze the hydrolysis of oseltamivir (3). As expected, sample 6 (lane 6) contained the lowest HCE1 protein and showed the lowest hydrolytic activity toward oseltamivir. Conversely, sample 7 (lane 7) contained the highest level of HCE1 protein and was the most active toward this anti-viral

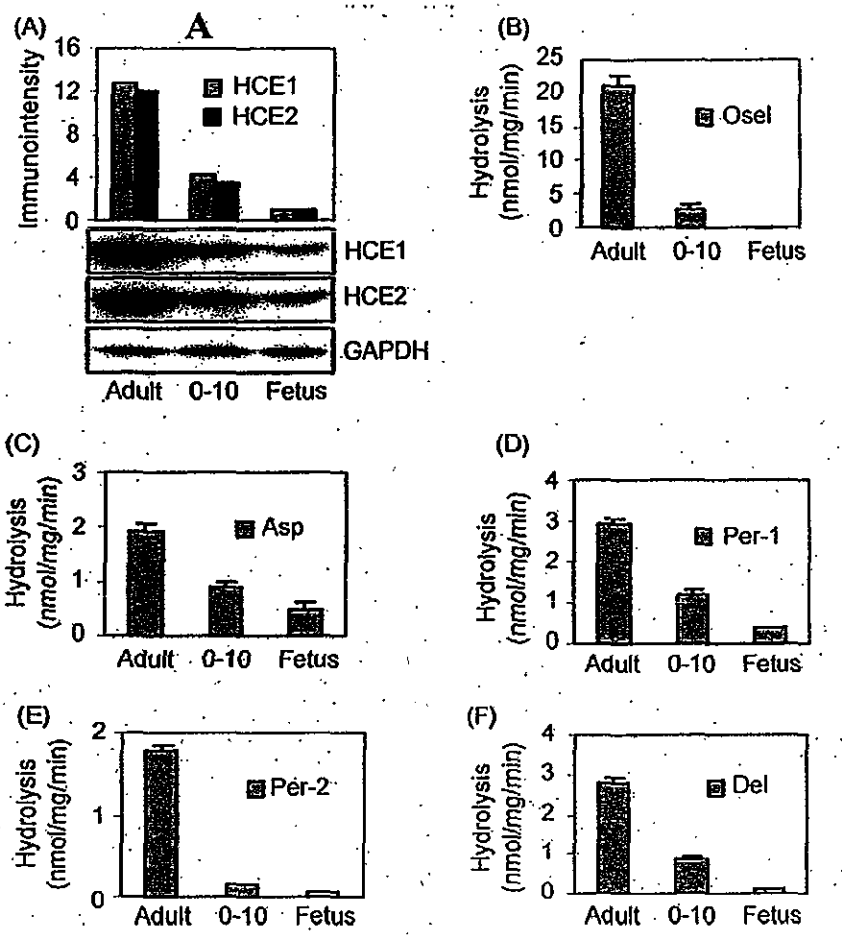

Fig. 4 – Hydrolysis of oseltamivir, aspirin, deltamethrin and permethrin. (A) *Western analysis of pooled samples for various groups.* Microsomes (1.5 μg) pooled from the adult, child and fetal group were analyzed by Western blotting and the immunostaining intensities were quantified by the KODAK 1D Image Analysis Software. (B) *Hydrolysis of oseltamivir by pooled microsomes.* Microsomes (20 μg) from various age groups were incubated with oseltamivir (200 μM) at 37 °C for 10 min, and the formation of oseltamivir carboxylate was detected by LC–MS/MS. (C) *Hydrolysis of aspirin by pooled microsomes.* Microsomes (80 μg) from various age groups were incubated with aspirin (1 mM) at 37 °C for 60 min, and the formation of salicylic acid was detected by HPLC. (D and E) *Hydrolysis of permethrin by pooled microsomes.* Microsomes (50 μg) from various age groups were incubated with permethrin (100 μM) at 37 °C for 60 min, and the disappearance of the parent compounds was detected by HPLC. Permethrin contained a mixture of *cis*- and *trans*-isomers at a ratio of 46 and 52%, respectively with the *cis*-form having a retention time of 10.41 and the *trans*-form of 10.18. (F) *Hydrolysis of deltamethrin by pooled microsomes.* Microsomes (50 μg) from various age groups were incubated with deltamethrin (100 μM) at 37 °C for 60 min, and the disappearance of the parent compound was detected by HPLC. Data were assembled from three independent experiments with each experiment having two injections.

agent (Fig. 3A and C). The hydrolysis of oseltamivir among these individual samples was highly correlated with the protein level of HCE1 with a correlation coefficient of 0.9373, although samples 8 and 10 exhibited relatively lower hydrolysis compared with their relatively HCE1 contents (Fig. 3A and C).

3.3. *Hydrolysis of drugs and insecticides by liver microsomes of fetuses, children and adults*

We next extended the metabolism study to include the anti-inflammatory agent aspirin, and insecticides permethrin (*cis*- and *trans*-) and deltamethrin. In addition to their pharmacological and toxicological implication, these chemicals were chosen because they are hydrolyzed in an isoform-specific manner. Oseltamivir and deltamethrin are predominately hydrolyzed by HCE1 [3,19,20], whereas aspirin is predominately hydrolyzed by HCE2 [4]. The *cis*-form of permethrin is favorably hydrolyzed by HCE2, whereas the *trans*-form is comparably hydrolyzed by both forms. The extended metabolism study was performed with microsomes pooled from various age groups, and the microsomes were so pooled by mixing equal amount from all individuals in an age group. The pooled samples enabled comparison to be made on the overall hydrolysis of these chemicals among different age groups.

We first determined the levels of HCE1 and HCE2 in the pooled samples by Western immunoblotting. Based on the immunostaining intensities, the child group expressed ~25%

of carboxylesterases (both HCE1 and HCE2) of the adult group and the fetal group expressed less than 10% of the adult group (Fig. 4A). Overall, the magnitude of hydrolysis of these chemicals was correlated well with the relative levels of carboxylesterases in these samples (Fig. 4B–F). The adult pooled sample showed the highest activity toward all chemicals and the fetal pooled samples showed the lowest activity. Hydrolysis of aspirin and *trans*-permethrin (Per-1) by the samples pooled from the fetuses and children was slightly higher than that predicted according to their relative abundance of carboxylesterases. For example, the fetal sample contained less than 10% of carboxylesterases of the adult sample but showed 25% of aspirin hydrolysis (Fig. 4A and C). Conversely, the hydrolysis of oseltamivir, *cis*-permethrin and deltamethrin by the fetal group was less than 10% of the adult group. The precise mechanism on these discrepancies remains to be determined. On the other hand, the higher-than predicted hydrolysis, in the case of aspirin, was likely due to hydrolysis by other enzymes (highly expressed in the fetal liver) or due to polymorphic variants. In support of these possibilities, butyrylcholinesterase has been shown to hydrolyze aspirin [21], and certain polymorphic variants of HCE2 were found to differ from the wild-type enzyme in hydrolyzing this anti-platelet agent [4]. In addition, the liver expresses a third carboxylesterase [22], but it remains to be determined whether this carboxylesterase hydrolyzes these compounds (i.e., aspirin and *trans*-permethrin) and whether the expression of this carboxylesterase is developmentally regulated.

4. Discussion

Carboxylesterases constitute a class of hydrolytic enzymes that play important roles in the metabolism of therapeutic agents and detoxication of insecticides [1]. In this study, we analyzed a large number of individual liver samples for the expression patterns of HCE1 and HCE2, two human carboxylesterases predominately expressed in the liver. Overall, the adult group expressed significantly higher HCE1 and HCE2 than the child group or the fetal group. The age-related expression was confirmed on the levels of both mRNA and protein. In agreement with the expression patterns, the adult microsomes were approximately 4 times as active as the child microsomes and more than 10 times as active as the fetal microsomes in hydrolyzing a group of therapeutic agents and insecticides. Even within the same age group, a large inter-individual variability was detected in mRNA and protein levels as well as hydrolytic activity.

Although both HCE1 and HCE2 exhibited a similar expression pattern among the various age groups, there were several major differences. First, HCE1 exhibited much greater inter-group and inter-individual variability than HCE2. For example, based on the values of the means, the adult group displayed a 319-fold higher level in HCE1 mRNA compared with the fetal group. In contrast, these two groups showed only a 55-fold difference in the level of HCE2 mRNA (Fig. 1 and Table 2). Likewise, the adult group showed a 430-fold inter-individual variability in HCE1 mRNA (ratio between maximum over minimum), in contrast, only a 21-fold difference in HCE2 mRNA was detected in the same group (Table 2). Second, HCE1 displayed better age-related expression than HCE2 in the child group with correlation coefficients of 0.3138 and 0.1058, respectively (Fig. 2C and D). In contrast, HCE2 displayed better correlation in the fetal group with correlation coefficients of 0.4362 and 0.1136, respectively (Fig. 2A and B).

The large inter-individual variability, particularly in the fetal and children groups, was likely an outcome coordinated by multiple mechanisms. In this study, we have shown that the adult group expressed the highest levels of HCE1 and HCE2 followed by the child group, and the fetal group expressed the lowest levels of both enzymes (Fig. 1 and Table 2). Such age-related expression patterns were confirmed by RT-qPCR and Western analyses (Figs. 1, 3 and 4) and established that developmental regulation is involved in the expression of HCE1 and HCE2. However, the correlation with age in many cases was only moderate at the most and did not reach the levels of statistical significance (Fig. 2C and F). Although the precise mechanisms remain to be determined, the lack of strong correlation with age in these groups was likely due to complicated factors such as the administration of therapeutic agents and disease conditions. We have previously reported that pathological condition and therapeutic agents markedly altered the expression of HCE1 and HCE2 [6,8]. Interleukin-6, a cytokine usually elevated during inflammation, profoundly suppressed the expression of both HCE1 and HCE2 [8]. Consistent with the suppression of carboxylesterases by cytokines, patients with elevated cytokine conditions such as liver cirrhosis had much lower capacity of hydrolyzing ester drugs such as perindopril, a non-sulphydryl angiotensin converting enzyme inhibitor [23,24].

The significantly lower level of HCE1 in the child group, compared with the adult group, provides a molecular explanation to the large pharmacokinetic difference in oseltamivir between these two groups. In this study, the sample pooled from the children was only ~15% as active as the sample pooled from the adults in hydrolyzing oseltamivir (Fig. 4C). Consistent with the in vitro metabolism, children under 12 years old reportedly produced only approximately half of the hydrolytic metabolite produced by adults [25]. Apparently the low level production of oseltamivir carboxylate in children was likely due to ineffective hydrolysis of the parent drug and higher clearance of the metabolite. Furthermore, we have shown that individual samples in the child group varied by as many as 127-fold in oseltamivir hydrolysis (Fig. 4A). Pharmacokinetic studies in children, however, did not detect such a large inter-individual variation in the production of hydrolytic metabolite of oseltamivir [25,26]. One explanation is that the frequency with an extremely low expression level of HCE1 is rare in the general population, and the pharmacokinetic studies were performed in 24 or fewer children [25,26]. Indeed, there was a reported rare case that an adult patient with diabetes mellitus had only 1–2% capacity of normal people in hydrolyzing clopidogrel based on the values of the means and standard deviations [27]. Like oseltamivir, clopidogrel is a substrate of HCE1 [3]. Ineffective hydrolysis of oseltamivir, on the other hand, likely leads to increased concentration in the brain. Some patients taking oseltamivir reportedly developed neurobehavioral changes [28], although a direct link remains to be established between the developed neurotoxiciy and the use of oseltamivir.

In contrast to oseltamivir, pyrethroids have long been recognized to exert neurotoxicity [29]. As a class of the most used insecticides in the world, both the general population and workers have a high risk to be exposed to these insecticides. Epidemiological studies have shown that the exposure level, in some cases, can be high [30,31]. Pyrethroid insecticides are generally considered safe to mammals, because they are rapidly eliminated by carboxylesterases. In this study, we have shown that the fetuses and children hydrolyzed pyrethroids at a rate of only ~20% or lower of the adults (Fig. 4D–F), suggesting their vulnerability to pyrethroids-induced toxicity. In support of this notion, neonatal rats were reportedly 17 times as sensitive as adult rats to cypermethrin [12]. Neurotoxicity induced by pyrethroids appears to cause irreversible damage. Prenatal exposure to deltamethrin, for example, led to a deficit in locomotor activity of offspring post-natally at 9 weeks [32]. In humans, micromolar concentrations were reported in the meconium [33]. This is particularly of relevance as fetuses have only limited capacity of hydrolytic detoxication as described in this report.

In summary, our work points to several important conclusions. First, the expression of both HCE1 and HCE2 increases with age, establishing that their expression is developmentally regulated and that fetuses and children generally have lower capacity of hydrolytic metabolism than adults. Second, there is a large inter-individual variability in the expression of these enzymes, particularly in the fetal and child groups. It is likely that the expression of HCE1 and HCE2 in these age groups is subjected to non-developmental regulation with high sensitivity (e.g., xenobiotic regulation). Carboxylesterases are recognized to play important roles in drug metabolism and insecticide detoxication. The findings on the large variability among different age groups or even within the same age group have important pharmacological and toxicological implications, particularly in relation to altered pharmacokinetics of ester drugs in children and vulnerability of fetuses and children to insecticides such as pyrethroids.

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A−05 異常行動モデルとしての薬物誘発ジャンピング行動に対するタミフルの影響とその防御法に関する研究

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【背景と目的】タミフルを代表とする抗インフルエンザウイルス剤服用者が、異常行動の結果、事故死を起こしたことが報告され、緊急安全性情報が出された。その後この因果関係に関しては国家的研究班が組織され、調査研究が行われているが、詳細は未だ明らかでない。しかし、世界的に見れば新たなインフルエンザウイルスの出現もあり、それらの対処は急がねばならない状況と思われる。このような状況を踏まえ、我々は新たな視点から抗インフルエンザウイルス薬の欠点を補う可能性を検討した。

【実験方法】ジャンピング行動は、直径32 cmの正八角形、高さ35 cmの台に置いたマウスが薬物投与後40分間に下の床に飛び降りる行動とし、飛び降りるまでの時間と回数を（飛び降りた動物は台に戻し）測定した。尚、マウスは測定までこの測定台へ置かれたことはない。薬物の投与スケジュールは、haloperidol 0.5 mg/kg (ip) 5分後にclonidine 10mg/kg (ip)投与し、測定台へ移し測定を開始した。タミフル0.1%CMC懸濁液はhaloperidol 15分前に経口投与し、前処置薬はタミフルのさらに10分前にip投与した。

【結果並びに考察】タミフル150mg/kg、haloperidol、clonidineそれぞれ単独投与では測定時間内にジャンピング行動は全く起こさなかった。haloperidolとclonidineの併用では、0.63回/匹のジャンピング行動が見られた。このhaloperidol-clonidine誘発行動は、タミフル150mg/kgの併用により22.6回/匹と有意に増強された。このタミフルによる増強作用は、acetazolamide 20 mg/kg前処置により0.50回/匹と有意に抑制し、150 mg/kg前処置ではジャンピング行動を完全に消失させた。さらに、diazepam 0.5、1 mg/kg、valprorate 40 mg/kg、fluoxetine 5 mg/kg前処置により、タミフルによる薬物誘発ジャンピング行動は消失した。したがって、このようなジャンピング行動は、異常行動の一つの指標として有用と考えられる。現時点ではこれらの行動の発現機序は不明であるが、各種中枢神経伝達物質系の不均衡が関与し、抑制作用はその是正によることが示唆される。

A−06 Morphine誘発精神依存形成におけるL型高電位開口性カルシウムチャネル（HVCCs）機能亢進に対するPI 3-kinaseの関与

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【目的】我々は既に、精神依存の評価法である条件づけ場所嗜好性試験において、L型HVCC拮抗薬であるnifedipineにより、morphine誘発報酬効果が有意に抑制されることを報告した。一方、PI 3-kinase class IIIであるVps34はtraffickingに深く関与することが報告されていることから、morphineによるL型HVCCの発現増加に関与している可能性が考えられる。そこで本研究では、morphineによる精神依存形成過程におけるL型HVCCの発現増加を伴った機能亢進機序を、精神依存マウスおよび依存性薬物を連続曝露した初代培養大脳皮質神経細胞（神経細胞）を用いて、行動薬理学的および神経科学的観点から検討した。

【方法】Morphineによる報酬効果は条件づけ場所嗜好性試験により行った。神経細胞へのmorphineの連続曝露は、Hanks液で希釈したものを直接培養液中に添加した。[$^{45}Ca^{2+}$]$_i$流入は2分間の30 mM KCl刺激により神経細胞内へ取り込まれた放射活性を測定した。蛋白発現量はWestern blot法により解析した。

【結果および考察】Morphineによる報酬効果は、L型HVCC阻害薬（nifedipine）の前処置により完全に消失した。この時点での側坐核を含む領域および大脳皮質画分におけるL型HVCC α1cおよびα2/δ subunitならびにVps34蛋白の発現量に有意な増加が認められた。同様に、神経細胞にmorphineを連続曝露した場合に観察される30 mM KCl誘発[$^{45}Ca^{2+}$]$_i$流入の増加は、L型HVCC阻害薬の併用により完全に阻害された。また、morphineの連続曝露によりL型HVCC α1cおよびα2/δ subunitならびにVps34蛋白量の有意な増加が認められた。このα1c subunit蛋白量の増加は、PI 3-kinase阻害薬（LY294002）の併用処置により有意に減少した。以上の成績より、morphineによるL型HVCC α1c subunit蛋白発現増加機構に、Vps34が一部関与することが明らかとなった。

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