J. Biochem. **144,** 685–690 (2008)
doi:10.1093/jb/mvn122

Expression and Secretion of *N*-acylethanolamine-hydrolysing Acid Amidase in Human Prostate Cancer Cells

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Received July 14, 2008; accepted September 8, 2008; published online September 19, 2008

***N*-acylethanolamines (NAEs) are a class of bioactive lipid molecules in animal tissues, including the endocannabinoid anandamide and the anti-inflammatory substance *N*-palmitoylethanolamine. Enzymatic hydrolysis of NAEs is considered to be an important step to regulate their endogenous levels. Lysosomal NAE-hydrolysing acid amidase (NAAA) as well as fatty acid amide hydrolase (FAAH) is responsible for this reaction. Here, we report relatively high expression of NAAA in human prostate cancer cells (PC-3, DU-145 and LNCaP) and prostate epithelial cells (PrEC), with the highest mRNA level in LNCaP cells. FAAH and the NAE-forming enzyme *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) were also detected in these cells. NAAA activity in LNCaP cells could be distinguished from coexisting FAAH activity, based on their different pH dependency profiles and specific inhibition of FAAH activity by URB597. These results showed that both the enzymes were functionally active. We also found that NAAA was partly secreted from LNCaP cells, which underlined possible usefulness of this enzyme as a biomarker of prostate cancer.**

Key words: *N*-acylethanolamine, anandamide, endocannabinoid, LNCaP cell, lysosomal enzyme.

Abbreviations: FAAH, fatty acid amide hydrolase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; NAAA, *N*-acylethanolamine-hydrolysing acid amidase; NAE, *N*-acylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D; NOE, *N*-oleoylethanolamine; NPE, *N*-palmitoylethanolamine; TLC, thin layer chromatography.

N-acylethanolamines (NAEs) are ethanolamides of long-chain fatty acids and have received considerable attention due to their biological activities in animal tissues (*1–3*). In particular, *N*-arachidonoylethanolamine (anandamide), *N*-palmitoylethanolamine (NPE) and *N*-oleoylethanolamine (NOE) have been extensively studied as an endogenous ligand of cannabinoid receptors (endocannabinoid), an anti-inflammatory substance, and an anorexic substance, respectively (*1–3*). In animal tissues, the major pathway to degrade NAEs is enzymatic hydrolysis to free fatty acids and ethanolamine. The enzymes fatty acid amide hydrolase (FAAH) and NAE-hydrolysing acid amidase (NAAA) are known to be responsible for this reaction. FAAH is a well-characterized membrane-bound serine hydrolase, while NAAA is a lysosomal hydrolase with homology to acid ceramidase (*4*). Since this reaction is considered to be a critical step for the regulation of endogenous NAE levels (*5*), it should be important to clarify tissue distribution of these enzymes in human tissues and characterize the native enzymes. We previously reported the presence of NAAA in cell lines derived from human blood cells (megakaryoblastic cells CMK and macrophage-like cells U937 and THP-1) (*6*, *7*). However, NAAA of other human tissues has been poorly understood. In the present study, we report for the first time relatively high level of NAAA expression in human prostate.

MATERIALS AND METHODS

Materials—[1-^{14}C]Palmitic acid (2.06 GBq/mmol) was purchased from PerkinElmer Life Science (Boston, MA, USA); [1-^{14}C]arachidonic acid (2.07 GBq/mmol), horseradish peroxidase-linked anti-rabbit IgG, Hybond-P and an ECL Plus kit were from GE Healthcare Life Sciences (Piscataway, NJ, USA); palmitic acid and BSA were from Sigma (St Louis, MO, USA); arachidonic acid was from Nu-Chek-Prep (Elysian, MN, USA); NPE and URB597 were from Cayman Chemical (Ann Arbor, MI, USA); dithiothreitol (DTT) was from Wako Pure Chemical (Osaka, Japan); ethanolamine and Nonidet P-40 were from Nacalai Tesque (Kyoto, Japan); *n*-octyl-β-D-glucoside was from Dojindo (Kumamoto, Japan); random hexamer and *Ex Taq* DNA polymerase were from Takara Bio (Ohtsu, Japan); protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA, USA); human MTC panel I and II cDNAs were from Clontech (Mountain View, CA, USA); pre-coated silica gel 60 F_{254} aluminum sheets for TLC (20 × 20 cm, 0.2 mm thickness) were from

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Merck (Darmstadt, Germany); Trizol, Moloney murine leukemia virus-reverse transcriptase, RPMI 1640 and fetal calf serum (FCS) were from Invitrogen (Carlsbad, CA, USA); prostate epithelial basal cell medium was from BioWhittaker Inc. (Walkersville, MD, USA); Amicon Ultra-15 centrifugal filter devices were from Millipore (Billerica, MA, USA); a human prostate epithelial cell line (PrEC) was from Clontics (Walkersville, MD, USA); and prostate cancer cell lines (PC-3, Du-145, and LNCaP) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). [^{14}C]NPE and [^{14}C]anandamide were synthesized from ethanolamine and [1-^{14}C]palmitic acid or [1-^{14}C]arachidonic acid as described previously (*8*). Anti-NAAA polyclonal antibody was raised in a rabbit against a mixture of three peptides corresponding to residues 229–250 (LTESEDFEAAVYTLAKTPLIAD), 291–305 (TNYDHWEPVPKRDDR), and 346–362 (MSAA EPDKYMTMIRNPS) of rat NAAA (*9*).

Cell Culture—PC-3, DU-145 and LNCaP cells were cultured at 37°C in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM glutamine in a humidified 5% CO_2/95% air incubator. PrEC cells were cultured in prostate epithelial basal cell medium according to the manufacturer's instructions.

Enzyme Preparation—For the preparation of intracellular NAAA, the cells were cultured in the presence of 10% FCS in a 100 mm dish, harvested with the aid of trypsin, washed twice, suspended in 0.4 ml of PBS, and subjected to sonic disruption. The sample was then used as homogenate. The solubilized enzyme was prepared from the homogenate as described previously (*6*). Protein concentration was determined by the method of Bradford (*10*) with BSA as standard. For the preparation of released NAAA, the culture medium was replaced with serum-free medium containing 10 mM NH_4Cl, and the cells were further cultured for 36 h. The culture medium, into which NAAA was released, was then collected and centrifuged at 300*g* for 10 min twice to remove contaminated cells. The obtained medium was transferred into an Amicon Ultra-15 centrifugal filter device and concentrated 80 fold by centrifugation at 2,000*g*. During the concentration, the buffer was replaced with PBS containing 0.1% octyl glucoside. Recombinant human NAAA was transiently expressed in human embryonic kidney (HEK) 293 cells as described previously (*11*). All the samples were stored at −80°C until use.

RT–PCR—Total RNA was isolated with Trizol reagent from various human prostate cells. cDNAs were then synthesized by the use of Moloney murine leukemia virus-reverse transcriptase and random hexamer, and were subjected to PCR amplification by *Ex Taq* DNA polymerase. Primers and PCR conditions for human NAAA, FAAH, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (*7*). Primers used for human *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) were 5′-GCA ACCAGTCTCTGATGACAAGCAG-3′ (sense) and 5′-GCT TAAGCACTGGGAGTTCTTTGTCTAG-3′ (antisense). PCR conditions for NAPE-PLD were: denaturation at 94°C for 48 s, annealing at 65°C for 48 s and extension at 72°C for 48 s (30 cycles). To examine distribution of NAAA in various human organs, PCR was performed with human MTC panel I and II cDNAs as templates. PCR products, which were confirmed to be in the logarithmic phases, were electrophoresed on 1.6–1.8% agarose gels and stained with ethidium bromide.

Enzyme Assay—The enzyme was incubated with 200 μM [^{14}C]NPE or [^{14}C]anandamide (1000 cpm/nmol, dissolved in 10 μl of dimethylsulphoxide) at 37°C for 60 min (for the homogenates and solubilized proteins) or 120 min (for the concentrated culture medium) in 100 μl of 100 mM citrate–Na_2HPO_4 (pH 4.5) to detect NAAA activity or 100 mM Tris–HCl (pH 9.0) to detect FAAH activity. Both the buffers contained 3 mM DTT, 0.1% Nonidet P-40, 0.05% BSA and 150 mM NaCl. Separation by TLC and quantification of radioactive products were performed as described previously (*11*).

Western Blotting—After separation by SDS–PAGE on a 12% gel under denaturing conditions, proteins were electrotransferred to a Hybond-P membrane. The membrane was blocked with 5% non-fat dried milk in PBS containing 0.1% Tween-20 overnight, and then incubated with anti-NAAA antibody (1.8 μg/ml) in the blocking buffer at room temperature for 1 h, followed by the incubation with the horseradish peroxidase-linked secondary antibody (1:4000 dilution) in the blocking buffer at room temperature for 1 h. The immunopositive NAAA was then treated with an ECL Plus kit and detected with the aid of a LAS1000plus lumino-imaging analyzer (Fujix, Tokyo, Japan).

RESULTS

Expression of NAAA, FAAH and NAPE-PLD in Human Prostate—We first examined the expression level of mRNA for NAAA in various human tissues (Fig. 1A). Prostate showed the highest expression level, followed by leukocyte, liver, spleen, kidney and pancreas. The expression level was low or below detection limit with other tissues. To further analyse NAAA expressed in human prostate, we next examined expression of NAAA mRNA in prostate epithelial cells (PrEC) and prostate cancer cells (PC-3, DU-145 and LNCaP). As shown in Fig. 1B, NAAA was expressed in all these cell lines, with the highest mRNA level in LNCaP cells, followed by PrEC and DU-145 cells. PC-3 cells showed the lowest level. We also examined the other NAE-hydrolysing enzyme FAAH (*12*) and the NAE-forming enzyme NAPE-PLD (*13*, *14*) (Fig. 1B). LNCaP cells showed the highest expression level of FAAH mRNA, followed by DU-145. The mRNA was weakly expressed with PrEC, and hardly detected with PC-3 cells. We therefore used LNCaP cells for further analysis on NAAA and FAAH. On the other hand, NAPE-PLD mRNA was expressed in all of these cell lines at similar levels.

Co-localization of Catalytically Active NAAA and FAAH in LNCaP Cells—We investigated how NAAA and FAAH shared the NAE-hydrolysing activity in LNCaP cells. URB597 potently inhibited FAAH with an IC_{50} of 0.07 μM, but did not inhibit NAAA at concentrations at least up to 10 μM (*7*). In addition, NAAA showed the optimal pH around 4.5 and was almost inactive at

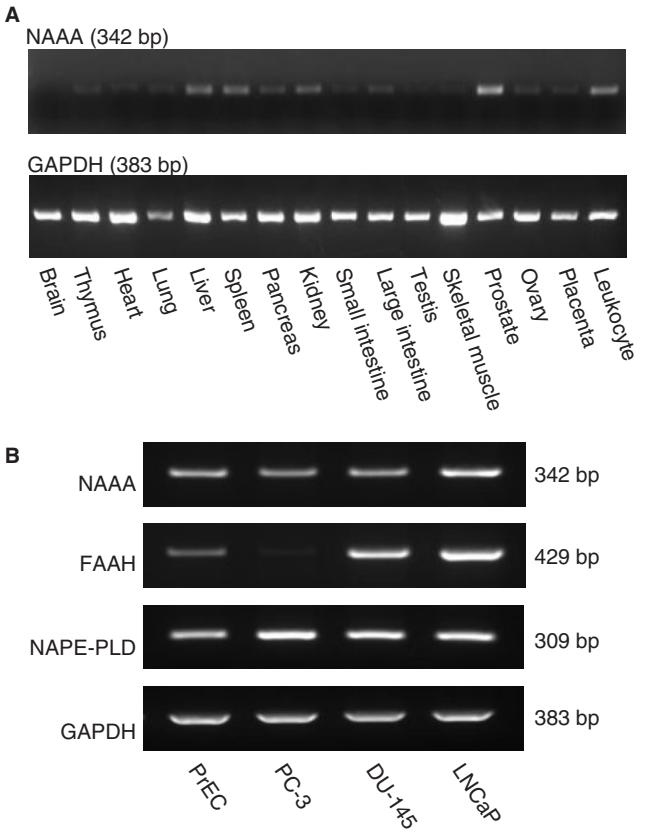

Fig. 1. **Expression of NAAA in human tissues and expression of endocannabinoid-related enzymes in prostate cells.** (A) The tissue distribution of NAAA mRNA in human was analysed by PCR. (B) Expression of mRNA for NAAA, FAAH and NAPE-PLD was examined by RT–PCR. GAPDH mRNA was used as a control.

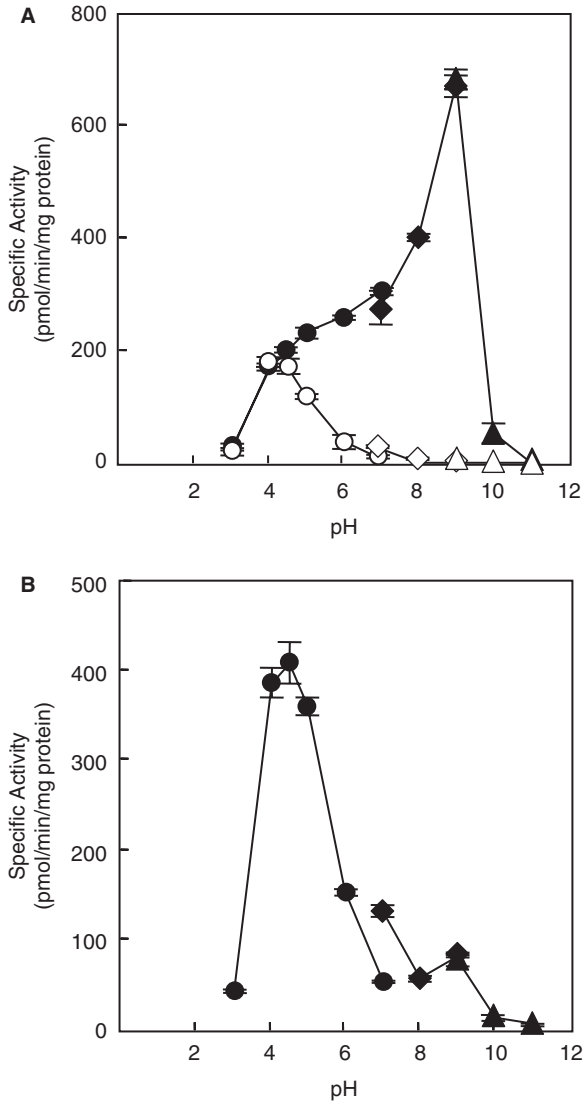

Fig. 2. **pH dependency of NPE-hydrolysing activity in LNCaP cells.** The homogenates (64 μg of protein) (A) or solubilized proteins (49 μg of protein) (B) of LNCaP cells were allowed to react with [^{14}C]NPE at the indicated pH in the presence of 10 μM of URB597 (open symbols) or in its absence (closed symbols). The pH was adjusted with the following buffers (100 mM): citrate–Na_2HPO_4 (circles), Tris–HCl (diamonds), Na_2CO_3–$NaHCO_3$ (triangles). Mean values ± SD are shown ($n = 3$).

neutral and alkaline pH (*6*, *15*), while FAAH was most active at pH 8.5–10 (*6*, *16*). The different pH-dependent activity profiles between NAAA and FAAH were successfully used to distinguish both the activities (*7*). We therefore examined NPE-hydrolysing activity of LNCaP cells at different pH in the presence or absence of 10 μM URB597. In the absence of URB597, LNCaP cell homogenates were active in a range of pH 3–10 with the highest activity at pH 9.0 (Fig. 2A). The addition of URB597 completely abolished the activity at pH 9.0. In contrast, URB597 hardly changed the activity at pH 4.5. Furthermore, NAAA, but not FAAH, was reported to be solubilized by freezing and thawing without detergent (*6*, *17*). As shown in Fig. 2B, even in the absence of URB597 the proteins solubilized from LNCaP cell homogenates by freezing and thawing revealed a pH dependent activity profile similar to that of the homogenate in the presence of URB597. These results clearly showed that LNCaP cells have functionally active NAAA as well as FAAH.

We also examined reactivity of the cell homogenate with anandamide (Fig. 3). At pH 9.0, the homogenate exhibited a higher hydrolysing activity for anandamide than that for NPE, both of which were sensitive to URB597. On the contrary, at pH 4.5 the homogenate was more active with NPE than with anandamide, and the anandamide-hydrolysing activity was more sensitive to URB597. The results suggested that the hydrolysis of anandamide is largely dependent on FAAH.

Release of NAAA from LNCaP Cells—Recently, we reported that recombinant human NAAA overexpressed in HEK293 cells is partly released into the medium (*11*). However, it remained unclear whether or not native NAAA is released. We therefore examined possible secretion of NAAA from LNCaP cells. When the culture medium was concentrated and subjected to enzyme assay, the NPE-hydrolysing activity was detected at pH 4.5 (Fig. 4A). This activity corresponded to about 2% of

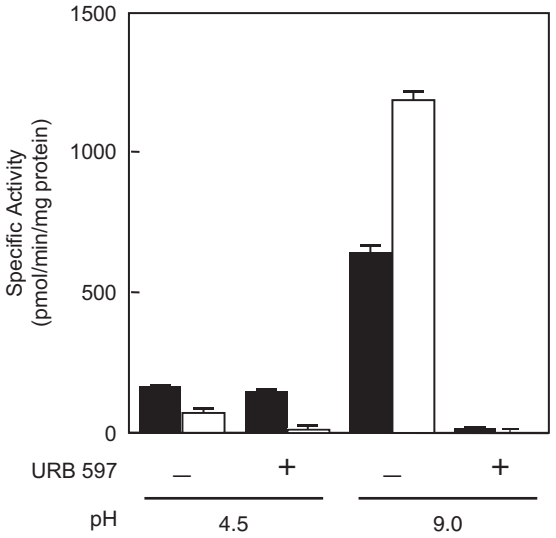

Fig. 3. **Reactivity of the LNCaP cell homogenates with NPE and anandamide.** The homogenates of LNCaP cells (48 μg of protein) were allowed to react with [^{14}C]NPE (closed columns) or [^{14}C]anandamide (open columns) at pH 4.5 or 9.0. URB597 at 10 μM was contained or not. Mean values ± SD are shown ($n = 3$).

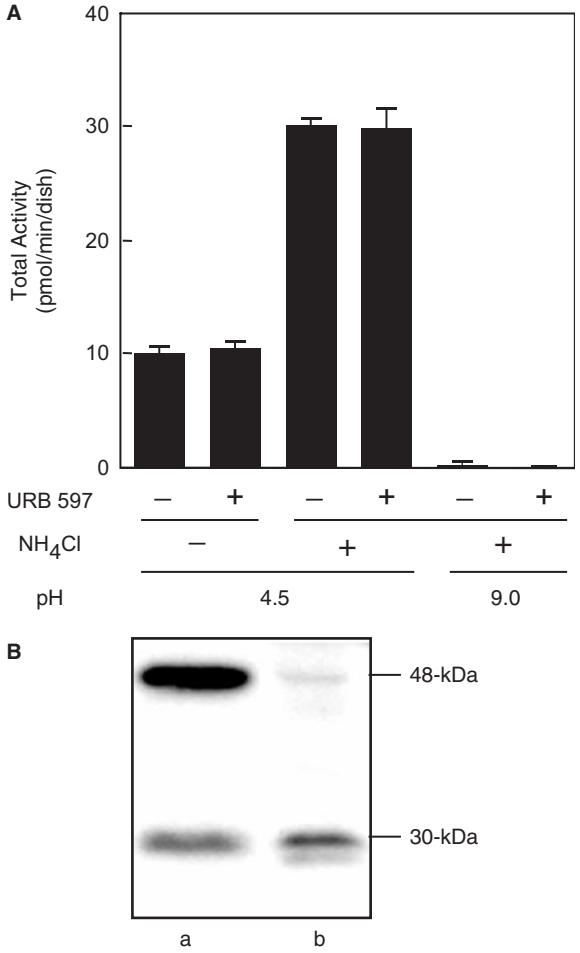

Fig. 4. **Secretion of NAAA from LNCaP cells.** (A) LNCaP cells were cultured for 36 h in the medium containing 10 mM NH_4Cl or not, followed by concentration of the culture medium. The concentrated medium (30 μl) was then allowed to react with [^{14}C]NPE at pH 4.5 or 9.0. URB597 at 10 μM was contained or not. Mean values ± SD are shown ($n = 3$). (B) The concentrated medium (20 μl) of LNCaP cells cultured in the presence of NH_4Cl (lane b) and recombinant human NAAA secreted from HEK293 cells (lane a) were analysed by western blotting with anti-NAAA antibody.

that in the cell homogenate. NH_4Cl is known to stimulate the secretion of lysosomal enzymes such as acid ceramidase and cathepsin B (*18*, *19*). When the cells were cultured in the presence of 10 mM NH_4Cl for 36 h, NAAA activity in the medium increased up to 3-fold. The activity at pH 4.5 was resistant to URB597 and the activity was hardly detectable at pH 9.0, showing that contamination of the membrane-bound FAAH, which might be derived from broken cells, was negligible. In addition, western blotting with anti-NAAA antibody revealed an immunopositive band around 30 kDa (lane b of Fig. 4B), which corresponded with the mature form of recombinant human NAAA overexpressed in HEK293 cells (lane a). Interestingly, this result with LNCaP cells was different from the NAAA-overexpressing cells which released the 48 kDa precursor as the major form of recombinant NAAA (lane a), in agreement with our previous report (*11*).

DISCUSSION

Although we previously reported tissue distribution of NAAA in mouse and rat, prostate has not been examined (*7*, *17*). In the present study, we revealed that in human tissues the prostate showed the highest NAAA mRNA level. Our results on tissue distribution of human NAAA were similar to that of human acid ceramidase-like protein (*20*), which was later shown to be identical with NAAA (*15*). However, prostate was not examined in the previous study. Furthermore, we exhibited expression of NAAA and NAPE-PLD in various human prostate cells for the first time. FAAH was also detected with prostate cells as reported by other researchers (*21*, *22*). We also exhibited that both NAAA and FAAH in LNCaP cells were catalytically active. In accordance with the reported substrate specificity of NAAA and FAAH (*15*, *16*), the former in LNCaP cells preferred NPE to anandamide, while the latter was highly active with anandamide.

NAAA is a lysosomal hydrolase degrading various NAEs with the highest reactivity toward NPE (*4*). Since the prostate expressed NAPE-PLD as a major NAE-synthesizing enzyme (Fig. 1B) and since prostate cancer cells contained endogenous anandamide (*21*, *22*), it was likely that NAEs are produced in this organ and degraded by NAAA as well as FAAH. Human prostate was reported to express the cannabinoid receptors CB1 and CB2, suggesting that anandamide acts as an endocannabinoid in this organ (*23*, *24*). NPE and NOE are present together with a lower level of anandamide in seminal plasma (*25*). Although NPE and NOE are inactive with cannabinoid receptors, they act as anti-inflammatory, anti-oxidant and antimicrobial agents,

suggesting their roles in protecting sperm against infection and maintaining sperm viability (*25*). Since the prostate secretes various substances into seminal fluid (*26*), NAEs detected in seminal plasma may be derived at least in part from prostate. Thus, NAE-related enzymes including NAPE-PLD, FAAH and NAAA of prostate may participate in the regulation of NAE levels in seminal plasma.

Endocannabinoids, including anandamide, its stable analogue (*R*)-methanandamide, and 2-arachidonoylglycerol, have been reported to show anti-proliferative effect on human prostate cancer cells (*24*, *27–29*). Moreover, alteration of FAAH level modulated invasion and migration of prostate cancer cells (*21*). Treatment of prostate cancer cells with NOE, which had been reported to be an inhibitor of acid ceramidase, led to the increase in intracellular ceramide levels, accompanied by an increase in apoptotic cells (*30*). NPE was also reported to possess anti-proliferative effect on cancer cells (*31*). *N*-stearoylethanolamine showed pro-apoptotic activity in rat C6 glioma cells (*32*). Considering these anti-tumor activities of NAEs, NAAA and other NAE-related enzymes expressed in prostate cancer cells may play a role in the regulation of cell growth.

LNCaP is a prostate cancer cell line established from metastatic lymph node, which responds to androgen stimulation for growth and hence represents an early and treatable cancer, while PC-3 and DU-145 are androgen-insensitive cell lines, which represent the androgen-refractory phase of advanced prostate cancer (*33*). Although LNCaP revealed the highest NAAA level, PC-3 and DU-145 showed NAAA levels similar to that of PrEC. Thus, it remained unclear if NAAA is down-regulated during loss of the antrogen-dependency.

High expression of lysosomal NAAA in prostate may be related to the fact that prostatic epithelial cells are enriched with lysosomes, which contain various lysosomal hydrolases including acid phosphatase, glucosidase and cathepsin B, L and D (*34*, *35*). In the present study, we found that NAAA is partly released into the medium (Fig. 4). The physiological role of the released NAAA is currently unclear. Like other lysosomal enzymes, the released NAAA may be catalytically active at inflammation sites where pH is acidic. Several secretory proteins such as prostate-specific antigen (PSA) received much attention as biomarkers of prostate cancer (*36–38*). NAAA was expressed not only in prostate cancer cells, but also in PrEC, normal prostate tissue and other human tissues (Fig. 1). Moreover, our previous studies showed the presence of NAAA in human megakaryoblastic cells CMK and macrophage-like cells U937 and THP-1 (*6*, *7*). Because it remains unclear if NAAA is also released from macrophages and other inflammatory cells, we can not rule out a possibility that extracellular levels of NAAA are elevated in human subjects with inflammatory diseases. Thus, further extensive studies will be required to clarify whether NAAA is a candidate to be developed as a prostate cancer-specific marker.

In conclusion, the present study revealed for the first time the expression of NAAA, a lysosomal enzyme hydrolysing bioactive NAEs, in prostate cancer cells. We also found that NAAA expressed in LNCaP cells is catalytically active and is partly secreted outside cells.

FUNDING

Ministry of Education, Culture, Sports, Science and Technology of Japan; Medical Institution Union Foundation; the Japan Foundation for Applied Enzymology; Kagawa University Specially Promoted Research Fund 2008.

CONFLICT OF INTEREST

None declared.

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