Perturbational Metabolic Profiling of Human Breast Cancer Cells

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Abstract

A major goal of toxicity testing is to obtain toxicity data for protecting public health and the environment from adverse effects that may be caused by exposure to environmental agents in the air, water, soil and food. The current toxicological studies that target human health effects primarily rely on animal studies. Unfortunately, large-scale animal exposure studies are expensive, requiring dedicated care and handling facilities. Furthermore, the US EPA and other organizations have adopted the goal of reducing animal usage in future toxicity testing programs.

One potential approach to increase the throughput of metabolomics is to use cell cultures instead of live animals in exposure studies, which provides obvious advantages. For example, there is no need to house and sacrifice animals, costs are significantly lower, and cells can be grown and exposed rapidly. Also, human cell lines can be employed in order to avoid cross-species extrapolations.

To conduct cell culture based metabolomics, we have developed a novel sample preparation method using adherent mammalian cells, which is rapid, effective, and exhibits greater metabolite retention by approximately a factor of 50 compared to the conventional sample preparation method.

We have applied this approach to study the metabolic changes caused by 17α-ethynyl-estradiol (EE2) in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines using NMR spectroscopy. Presented here are the details of sample preparation, NMR spectroscopy, metabolite identification, statistical analysis and intracellular metabolic changes.

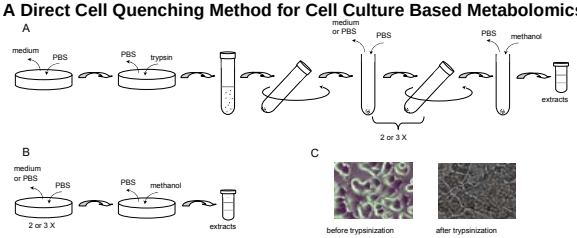
Introduction

One potential approach to increase the throughput of metabolomics is to use cell cultures instead of live animals in exposure studies. Although effective extrapolation to whole organism responses is ultimately required, such an approach provides obvious advantages such as low costs, shorter experimental time and no need to house and sacrifice animals. We have applied cell culture based metabolomics to study the metabolic changes caused by 17α-ethynyl-estradiol (EE2) in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines using NMR spectroscopy.

Estrogens are hormones that exert a wide range of physiological effects in reproductive, cardiovascular, musculoskeletal, immune and central nervous systems. Estrogens mediate their biological effects in target tissues by binding to the ER. ERα is an important predictive and prognostic marker in human breast cancer, being expressed in over 60% of cases. Binding of estrogens to ERα leads to transcriptional activation of ER target genes that are important in cell proliferation, angiogenesis and survival of breast cancer cells. In fact, certain types of breast cancers are thought to be possible consequences of exposure to environmental contaminants with estrogenic activity. EE2 was the first orally active pharmaceutical steroidal estrogen in contraceptive pills, and is now commonly used in the estrogen-progestin combination preparations. It is hormonally effective by activating the estrogen receptor.

Results and Discussion

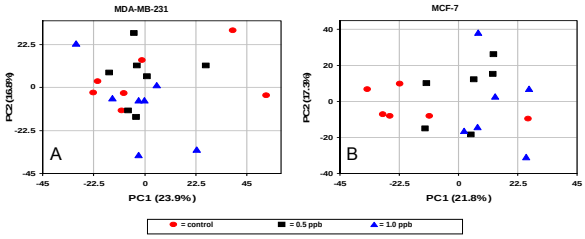
A Direct Cell Quenching Method for Cell Culture Based Metabolomics



(A) In the conventional quenching method for adherent cell cultures, cells are first detached from growth surfaces by trypsinization to obtain cell suspensions. Then, cells are centrifuged and washed with PBS for two or three times. The cells are then quenched with methanol (or other quench solutions) and extracted. (B) The new direct cell quenching method used in the study reported here. In this method, trypsin is not applied to the cells. After the culture medium is aspirated, cells are quickly rinsed with PBS twice. Residual PBS solution is removed by vacuum. The cells are then immediately quenched by methanol. Both cells and methanol solution are collected for the cell extraction.
(C) Images of MCF-7 cells before and after trypsinization. The appearances of the cells are significantly changed after trypsinization, indicating that the trypsin severely alters the physiological state of cells due to its interaction with membrane proteins.

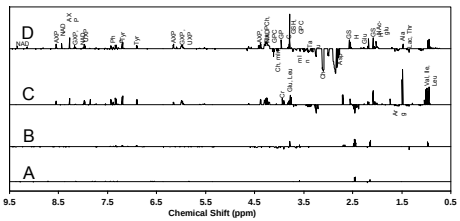
PCA Scores Plots

Polar Intracellular Extracts (control, 0.5 μg/L, and 1.0 μg/L EE2)



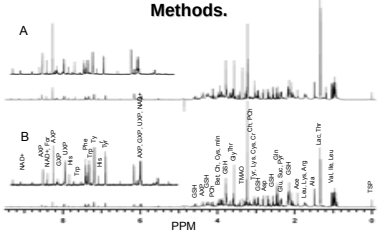
Scores plots of the first two principal components of PCA models built from the MDA-MB-231 and MCF-7 cell line exposure. (A)There is no discernable separation among the classes of MDA-MB-231 cells. (B) For the MCF-7 cells, the classes are reasonably well separated along PC1, which is the component that describes the maximum variation in the dataset. With the exception of one outlier, points for the control class are clustered to the left of the origin, while those for the 1.0 ppb exposure class are clustered to the right. Points for the 0.5 ppb class are mostly intermediate between the control and the high-concentration exposure classes. Therefore, PCA, which is a widely used and unsupervised multivariate method, is largely successful at capturing dose-dependent changes in metabolite profiles associated with the exposure for the case of ER-positive cells, but not for the case of ER-negative cells. This outcome demonstrates the effectiveness of our new method of quenching and harvesting cells for metabolomic studies.

T-test Filtered Difference Spectra

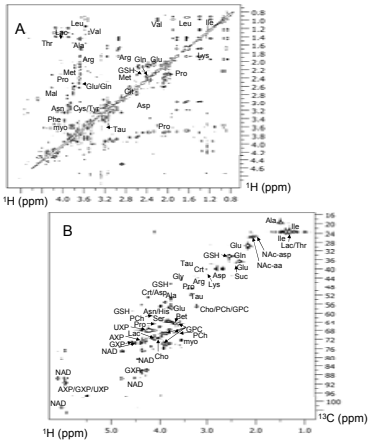


The "t-test filtered difference spectra" generated from binned ^{1}H NMR spectra of the aqueous extracts of cells following exposure to 17α-ethynylestradiol (EE2). Each difference spectrum was generated by subtracting the averaged bins of the relevant control class from those of each exposed class (A= MDA-MB-231, 0.5 ppb, B= MDA-MB-231, 1.0 ppb, C= MCF-7, 0.5 ppb, and D= MCF-7, 1.0 ppb). Positive peaks correspond to metabolites that increase upon treatment, whereas negative peaks are from metabolites that decrease.

Intracellular Metabolic Profiles of the Samples using Different Quenching Methods.



^{1}H NMR spectra of the aqueous extract of MCF-7 cells were obtained (A) using the conventional trypsinization/quenching method, or (B) using the new direct quenching method. The sample used for spectrum (A) had ~3x107 cells, whereas the sample in (B) contained ~6x105 cells. Both spectra were obtained using the same set of parameters (64 transients) and are displayed in the same vertical scale. The inserted spectra were also plotted using the same expanded y scale.



2D NMR spectra of MCF-7 intracellular extracts cultured in U-^{13}C glucose medium. (A) TOCSY and (B) gHSQC. Some assigned crosspeaks are labeled in the spectra.

Conclusion

We have developed a direct cell quenching method for metabolic profiling of cell cultures, which is rapid and effective. The method can also be used for MS-based *in vitro* metabolomics. Combined with an NMR sample automation system, the new method can be utilized for high throughput toxicity screening of chemicals or drugs. By using this direct cell quenching method, the study of metabolic changes caused by EE2 in ER-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cell lines demonstrated that the exposure of EE2 caused significant dose-dependent changes in the metabolite profiles of treated MCF-7 cells, but not with the MDA-MB-231 cells.